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From the Department of Histology, University of Upsala, Sweden
(Head: Professor Martin Wrete, M. D.)

ON THE FATE OF THE LYMPHOCYTE

BY

KARL-ERIK FICHTELIUS

BASEL (Schweiz) **S. KARGER** **NEW YORK**

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OF THE LYMPHOCYTE

BY

KARL-ERIK FICHTELIUS
MED. LIC., NORRL.

AKADEMISK AVHANDLING

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CHAPTER I

ABBREVIATIONS AND DEFINITIONS

Blood span of lymphocytes = the length of time during which the cells remain in the blood.

Central lymph (Yoffey and Drinker 1939) = lymph which has passed all lymph nodes, and will enter the blood stream without further change in composition.

DNA = Desoxyribose nucleic acid.

DNAP = Desoxyribose nucleic acid phosphorus.

Individual-specific antibodies (Simonsen 1953) = all antibodies that are produced in one animal against antigens from another animal of the same species (which the antibody-producer itself lacks).

Individual-specific antigen (Simonsen 1953) = a combination of different, genetically determined types of antigen characteristic of one individual organism.

Intermediate lymph (Yoffey and Drinker 1939) = lymph which has passed through one mass of lymphoid tissue, and may pass through another, before entering the thoracic duct.

H-cells = heterotransfused cells.

H spleen = rel. act. of DNAP in the spleen after heterotransfusion.

Life span of lymphocytes = the length of time during which the cells are morphologically and physiologically intact.

Lymphatic tissue (Hellman 1930) = solitary follicles + tonsils + splenic lymph nodules + lymph nodes.

Lymphoid tissue (Hellman 1930) = lymphatic tissue + bone marrow + thymus + diffuse lymphoid tissue in mucous membranes + red pulp of the spleen + diffuse lymhoid collections elsewhere.

Peripheral lymph (Yoffey and Drinker 1939) = lymph which has not yet passed through lymph nodes or come into contact with other collections of lymphoid tissue.

P = phosphorus.

P^{32} = radioactive phosphorus.

Rel. act. = relative activity (differently defined in different connections).

RES = reticulo-endothelial system.

S-cells = syngenesiotransfused cells.

Spec. act. = specific activity (differently defined in different connections).

Species-specific antigens (Simonsen 1953) = potentially antigenic characters which are common to all cells and protein-containing fluids in all individuals of a given species, and which therefore act as antigens only when introduced into another species.

S spleen = rel. act. of DNAP in the spleen after heterotransfusion.

CHAPTER II

INTRODUCTION

The lymphocytes are formed in the thymus and the lymphatic organs (see page 5). As shown by Andreasen and Ottesen (1945), the thymus is the most important seat of lymphopoiesis, except in old age when the weight of this organ is markedly reduced. Large numbers of lymphocytes are transported via the thoracic duct and the right lymphatic trunk into the blood. *The average length of time for which the lymphocytes remain in the blood* can be calculated (Bunting and Huston 1921, Yoffey 1933) from the number of lymphocytes daily entering the blood through the thoracic duct and the number normally present in the blood. This length of time is now considered to be 4 hours or less (Yoffey 1950), which means that either a circulation of lymphocytes exists, or the daily production and loss of lymphocytes is very large. It must be emphasized that the length of time during which the lymphocytes remain in the blood, the blood span, is not identical with the life span of these cells. No information is available concerning the life span of the lymphocytes under physiological conditions. We are awaiting a thesis on this subject by Ottesen (1953). He has made it probable that there are two kinds of lymphocytes: 1. lymphocytes which quickly leave the blood (unknown life span); 2. lymphocytes which can be indirectly demonstrated in the blood for nearly one year.

The question of the *disappearance of the lymphocytes from the blood* has not as yet been answered. There are, of course, several hypotheses. It has been supposed that many of the lymphocytes:

1. migrate through the intestinal epithelium and leave the organism via the lumen of the intestine (Bunting and Huston 1921);
2. return to lymphatic tissue whence they can re-enter the blood to start their cycle once more (Sjövall 1936);
3. are filtered out of the circulation in the bone marrow to serve as precursors of all other blood cells (Jordan and Speidel 1923);
4. disintegrate in the blood (Erf 1940);
5. become transformed into bone cells (Ökland 1940);

6. Become transformed into the epithelial cells of the epidermis (Andrew and Andrew 1949);

7. Are filtered out of the circulation in the lungs (Weisberger, Guyton, Heinle, and Storaasli 1951).

The problem of *the function of the lymphocyte* is intimately connected with the disappearance of the cells from the blood. The following list includes some of the connections in which lymphocytes and the lymphatic system are considered to be of importance.

1. The lymphocytes have a key position in the theories of blood formation. Monophylets look upon the lymphocyte as a temporary form of the haemocytoblast. The small, mature lymphocyte can, according to the extremists, be transformed first into a haemocytoblast, and later into the other cells of the blood. According to polyphylets the lymphocyte does not attain this multipotency (Downey 1938).

2. The participation of the lymphatic system in defence against infections was first suggested by Virchow (1860), and later on shown by many workers (Hellman 1919, Glimstedt 1936, and others). In this connection i. the ability to take up toxins has been ascribed to the lymphocyte (Bunting and Huston 1921, Sjövall 1936), ii. the lymphocyte has been considered to be a stem cell for the mononuclear phagocytes and other cells dominating the histology of inflammation (Maximow 1927—28, Bloom 1927—28, and others), iii. it has been proposed that lymphocytes are able to produce and to carry antibodies (Ehrich and Harris 1942, Haxthausen 1947, Wesslén 1952, and others).

3. Non-specific therapy, from the malaria treatment of Jaureg to ACTH and Cortisone, has a strikingly regressive effect on the lymphatic system (Selye 1946). The role of the lymphocytes in the physiology and pathology of connective tissue is not clear.

4. It has been thought that the lymphocytes have an important part to play in growth. The lymphocyte has been called a protein cell, analogous to the fat cell, by which the organism could transport concentrated proteins from one organ to another without any change in the colloid osmotic pressure of the blood (Andreasen 1939).

One of the approaches to the problem of the function of the lymphocyte, is, of course, to try to shed light on its behaviour in the healthy organism. An old dream among haematologists is to label

the lymphocyte. Many have tried, and in the last years labelling of lymphocytes with isotopes has come within the bounds of possibility. *This work* is concerned primarily with the disappearance of the lymphocytes from the blood. It is an attempt to develop a method by which this problem can be further studied. Radioactive phosphorus (P^{32}) has been used as a tracer, and the work has proceeded along two lines. Attempts have been made 1. to transfuse labelled lymphocytes from a radioactive donor to a non-radioactive recipient, and 2. to trace the labelled lymphocytes in one and the same animal without taking them out of the organism.

In the experiments on *transfusion of labelled lymphocytes*, large doses of P^{32} were administered to one or more animals, and a suspension of thymus-lymphocytes prepared. The radioactive lymphocytes were transfused to another animal, the organs of which were examined after 24 hours with regard to the concentration of P^{32}. The conclusions were based on a comparison of the distribution of radioactivity after syngenesiotransfusion (rat to rat, the same litter), heterotransfusion (rabbit to rat), and injection of inorganic P^{32}. After syngenesiotransfusion part of the radioactivity found in the spleen and liver was due to the injected lymphocytes, the fate of which differed from that of the heterotransfused cells, which were found in the liver. Similar transfusion experiments were performed on rats with aseptic peritonitis; the role of the spleen as a receptor of lymphocytes was confirmed, and new observations were made.

The labelling and tracing of lymphocytes in the same animal is based on the observations that lymphocytes labelled by a single injection of P^{32} seem to have a biphasic appearance in the blood, and the 'second top' of labelled lymphocytes can be produced prematurely if the animals are bled. The object of this experiment was to produce this second top earlier by bleeding, and then to examine possible receptor organs for radioactivity. A larger concentration of P^{32} was then to be expected at a given time in such receptor organs in the animals which had been bled than in those which had not. These experiments have so far yielded negative results.

CHAPTER III

THE FATE OF THE LYMPHOCYTE: REVIEW OF THE LITERATURE

INTRODUCTORY REMARKS

Before the extensive literature on the fate of the lymphocyte is summarized some fundamental questions must be discussed: 1. the size; 2. the developmental potency; 3. the probability of a circulation of the lymphocyte; 4. the identity of the lymphocytes from the thymus and the lymphatic organs.

THE SIZE OF THE LYMPHOCYTE. If lymphocytes of $< 10\ \mu$ are reckoned as small lymphocytes, 10—12 μ as medium, and $> 12\ \mu$ as large lymphocytes, then, according to Wiseman (1931—32), 83 % of the blood lymphocytes in supravital preparations are small. From this it is clear that when we consider the fate of the blood lymphocyte it is primarily the small lymphocyte with which we are concerned. Much confusion can be avoided if the difference is realized between the size and appearance of lymphocytes in supravital preparations and fixed smears. Wiseman found 83 % of small lymphocytes in supravital preparations but 20 % in fixed smears. He writes, 'In general, the tendency is decidedly toward an increase in the average diameter of the lymphocytes on fixation but no constant ratio of increase can be made out. It is probable that the cells which are thus swollen during fixation are the older and hence less resistent types, which would account for the number of large, pale forms in the fixed films. It may also be true that other younger cells shrink as an effect of the alcohol, increasing still more the discrepancy in cell size'.

DEVELOPMENTAL POTENCY. The question of the developmental potency of the lymphocyte has given rise to much controversy in the past, and has still not been resolved to the satisfaction of all. The

basic problem is whether the small lymphocyte is a mature cell or whether it is a primitive cell capable of growth and differentiation into monocytes, macrophages, fibroblasts, plasma cells, granulocytes, and erythroblasts (Yoffey 1950). During attempts to solve this problem the lymphocyte has been studied *in vivo* and *in vitro*, and histochemical methods have been applied.

In making any conclusions about the transformation of the small lymphocytes into other cell forms *in vitro* the following conditions must be fulfilled: 1. If culture is started with a colony of different types of cells, an individual lymphocyte must be observed; 2. the only way to avoid this trouble is to start with an absolutely pure colony of small lymphocytes. In similar experiments *in vivo* the individual lymphocyte must be followed. If a review on this subject were limited to experiments in which these conditions have been complied with it would be surprisingly short, embracing only a few experiments. The relation of monocytes to macrophages was studied by Clark and Clark (1930) in the transparent tails of amphibian larvae by means of injection experiments and observation of living cells in their normal environment. They observed the individual lymphocyte for 12 hours or more, and did not see any evidence of the transformation of small lymphocytes into monocytes or into any other type of cells. Ebert, Sanders, and Florey (1940) observed individual lymphocytes continuously for as long as 24 hours in transparent chambers in the rabbit ear. They found no sign of transformation into any other cell type.

It would be misleading to refer to only two experiments from the very extensive literature on this subject. Maximow and his pupil Bloom, who carried out studies *in vitro*, are among the most vehement in their defence of the multipotency of the small lymphocyte. In a work on culture of blood leucocytes, Maximow (1927—28) says, 'The lymphocytes and the monocytes of the mammalian blood *in vitro* are transformed in the course of a few hours into large amoeboid, phagocytic, dye-storing elements. These are identical in origin, structure, and function with the polyblasts or mononuclear exudate cells as found in inflamed tissue. . . . In their transformation into polyblasts the lymphocytes pass through a stage more or less similar to the monocyte'. Individual cells were, however, not observed, and the author started with a mixture of leucocytes. Perhaps the monocytes alone changed into polyblasts, and, even if it is probable that the large lymphocytes were also transformed, it is impossible to

come to any definite conclusion about the small lymphocytes after an experiment of this kind. The second advocate of the multipotency of the lymphocyte, Bloom (1927—28), states that when lymph from the thoracic duct 'is cultured *in vitro*, large and small lymphocytes change into typical inflammatory mononuclears or polyblasts which store lithium carmine'. Some large cells are always present in the thoracic duct lymph, and some of these are interpreted either as lymphocytes or monocytes by the various workers. Again, it is inaccurate to say that the small lymphocytes have developed in a certain way unless the individual cell has been observed. The conclusive demonstration of following an individual small lymphocyte over a period of several days and observing its growth into a polyblast or a large lymphocyte has yet to be made.

The cytological approach to the problem of the developmental potency of the small lymphocyte has contributed more to our knowledge than tissue culture. Wiseman (1931—32) confirmed by his supravital studies that the lymphocytes of the blood show the same changes in basophilia and mitochondrial content which accompany the maturation stages described for the other blood cells. In classifying the lymphocytes on the basis of these cytological criteria of age, the lymphocytes appearing in the normal blood can be divided into three main groups: 1. young (Y), 2. mature (M), and 3. old (O). In human and rabbit blood the average values for Y — M — O are 4.9 — 48.5 — 46.6 % of the total number of lymphocytes. Wiseman's observations seem to offer additional evidence in support of the hypothesis that at least a portion of the so called lymphocytes, arise, mature, function, and die as lymphocytes without differentiating into other cell types. Thorell (1945) analysed, by microspectrographical and microchemical methods, large, medium, and small lymphocytes in supravital preparations. 'The large lymphocytes have an endocellular nucleic acid metabolism indicating a high intensity of growth. The small lymphocytes, contrary to this, seem to have a very low intensity of growth'. The medium lymphocyte holds an intermediate position in this respect. The observations by Wiseman and Thorell accord well, and make the enlargement of the small lymphocytes and an increase in their nucleic acid metabolism less probable, but this possibility cannot be definitely excluded.

THE PROBABILITY OF A LYMPHOCYTE-CIRCULATION. Sjövall (1936) defends the old concept of Ehrlich (1891, quoted by Sjövall 1936), that a considerable part of the daily supply of lymphocytes to the blood is not a result of a true new formation within the lymphatic tissue. After repeated blood-letting in rabbits he found a fall in the blood lymphocyte count and a reduction of the lymphatic tissue. This seemed to indicate that the haemorrhagic losses constituted an increased strain on lymphopoiesis, but nevertheless no compensatory phenomenon was provoked. Lymphocyte-renewal would thus appear to be relatively inconsiderable, and the loss of cells seemed to be covered in part by the flushing out of free lymphocytes already present in the tissue. On the basis of this concept of the lymphatic tissue as a haematopoietic system with an inconsiderable normal rate of renewal, and the large daily supply of lymphocytes to the blood, Sjövall makes the following assumption, 'There is a "lymphocyte circulation" in the organism: from the lymphatic tissue, via the blood to the tissues out in the body and back to the place of origin'. This interpretation seemed natural to Sjövall in 1936 before Selye's description of the general adaptation syndrome (Selye 1946). Most of Sjövall's results can now be explained by the effect of stress.

Sjövall is criticized by Yoffey and Drinker (1939). Comparing the cell content of peripheral and central lymph (see page 5) they showed that of about 30 lymphocytes entering the blood via the thoracic duct only one underwent the cycle proposed by Sjövall. From this Yoffey and Drinker conclude that there is no rapid circulation of lymphocytes from blood to lymph. Their result does not, however, exclude the possibility that the lymphocytes leave the blood in the lymph nodes, and enter the intermediate or central lymph without ever having been in the peripheral lymph.

The importance of thymus and various lymphatic organs as a source of blood lymphocytes was studied by determining the rate of desoxyribose nucleic acid formation in the organs under examination (Andreasen and Ottesen 1944). They concluded that 'the lively lymphocytopoiesis in the lymphoid organs, above all in the thymus — — — — — goes decidedly against the notion of a lymphocyte circulation'.

However, the discovery by Ottesen (1953) that labelled lymphocytes can be shown indirectly in the blood for nearly one year (see page 7) is very confusing. How can this be reconciled with the

short blood-span of the lymphocyte without supposing a circulation of these lymphocytes from blood to some unknown organ and back again to blood? If most of the lymphocytes entering the blood were newly formed cells with a bloodspan of 4 hours, and were not returning to the blood from elsewhere, the same degree of maturity could be expected in all the lymphocytes of the blood. Wiseman (1931—32) and Thorell (1945), however, described lymphocytes of different degrees of maturity (see page 12). Their statements accord well with the hypothesis of a circulation of at least some part of the lymphocytes.

IDENTITY OF LYMPHOCYTES FROM THYMUS AND LYMPHATIC TISSUE. The lymphocytes in the blood are derived from thymus and from the lymphatic tissue (Kindred 1942, Andreasen and Ottesen 1945, Andreasen and Christensen 1949). Many investigations have been carried out to compare the lymphocytes in thymus and the lymphocytes in the blood. Most histologists nowadays agree that there are no significant differences between these cells (Whitney 1928, Hammar 1936, Drinker and Yoffey 1941, Bargmann 1943). Uncertainty, however, has arisen as to whether thymocytes can indeed be lymphocytes, since the thymus sometimes reacts differently from lymphatic tissue (Wiseman 1931, von Albertini, Gasser, and Wuhrmann 1935—36, Harris *et al.* 1948, Warren, McMillan, and Dixon 1950).

The most clear-cut information is to be obtained from a direct comparison between lymphocytes from thymus and from lymphatic organs, avoiding the mixture of lymphocytes of different origin in the blood. Such a comparison cannot be made with the cells *in situ*, because it is impossible to exclude environmental influences. As an example the reader is referred to an investigation by Smith and Thomas (1950). Glycogen was found in the small cortical lymphocytes of thymuses of kittens and mice, but no lymphocytes containing glycogen were to be found in the lymph nodes. This difference can be explained according to Dempsey and Wislocki (1944, quoted by Smith and Thomas 1950), as an effect of a relatively poor oxygen supply in the small lymphocytes of the cortex of the post-natal thymus. Smith and Thomas thus conclude that their findings seem to be due to structural differences in the organs, and not to variations in the lymphocytes.

To avoid such trouble of interpretation the lymphocytes must be

removed from the organs where they are formed, and then compared by suitable tests. Thymus lymphocytes have been studied *in vitro* (Tschasswnikow 1926, Emmart 1936, and others), and they behave exactly as other lymphocytes. However, to make conclusions about the functional identity of cells from *in vitro* studies may be very misleading; tests *in vivo* are called for. Following injection of large quantities of homologous thymocytes into the blood stream of rabbits, accumulations of lymphocytes were found in the liver and spleen (Osogoe and Hitachi 1950). Similar accumulations were found after homologous transfusion of lymphocytes (Osogoe 1950). According to Osogoe and Hitachi this proves the physiological identity of thymocytes and lymphocytes. The experiment will be further discussed on page 20.

Summarizing the above it can be said that so far no indisputable differences have been shown between lymphocytes from the thymus and from lymphatic tissue.

METHODS OF APPROACH TO THE PROBLEM

HISTOLOGICAL STUDIES. *The blood.* There is no doubt that some disintegrating lymphocytes are occasionally found in the blood. Wiseman (1931—32), already referred to in page 12, describes the end-stage of the lymphocyte as a dead, degenerating cell, constituting 5 % of the total lymphocytes in the blood. In view of the rapid rate of disappearance of lymphocytes from the blood, far more of these end-stages should be seen if senescence and degeneration were the main reason for the lymphocyte disappearance (Drinker and Yoffey 1941).

That the lymphocytes probably leave the blood was shown by Bunting and Huston (1921). They isolated between double ligatures a section of the jugular vein in rabbits. Cell counts in this jugular blood were made six hours later in some of the animals and twenty-four hours later in others. In stained films from the jugular blood the lymphocytes did not show any tendency towards disintegration, but were as sharply stained and defined as in films from the freely circulating blood. The authors conclude, 'They must leave the blood stream'.

Epithelium of the digestive and respiratory tract. Many authors have found that large quantities of lymphocytes migrate through the

epithelium of the digestive and respiratory tracts at different sites, especially in the tonsils and small intestine. Reviews of the literature are given by Stenqvist (1934), Andrew and Sosa (1947), and Andrew and Burns (1947), among others. Stenqvist criticizes some of the earlier investigations, and himself concludes that the lymphocytes do not migrate through the intestinal epithelium into the lumen. The contradictory results can be explained by damage to the epithelium during histotechnical preparation. This criticism also applies to an experiment by Jassinowsky (1925), who examined the cells in the washings from a piece of isolated intestine. By fixing the intestines *in situ* Stenquist avoided such artefacts. He found, like many others, that the lymphocytes only to a minor extent are situated above the level of the nuclei of the epithelial cells. Like Hellman (1932), who examined the tonsils, he supposes that the lymphocytes constitute a wall of defence against vegetating bacteria in the lumen. This hypothesis has been strongly upheld by Glimstedt (1933), who examined bacteria-free animals. Opinions still diverge about the importance and magnitude of the supposed migration of lymphocytes through the epithelium of the mucous membranes. This problem can hardly be conclusively solved by the classic, morphological methods.

Epidermis. As in the epithelium which covers the inner surfaces of the body, lymphocytes occur constantly in the normal epidermis. Recent reviews of the literature are given by Andrew and Andrew (1949), and Andreasen (1952). There is general agreement that lymphocytes are not lost on the surface of the epidermis after migration through it. However, the ultimate fate of the lymphocytes in epidermis is not clear. Andrew and Andrew write that there is nothing to indicate that lymphocytes degenerate within the epidermis, but that the lymphocytes are transformed into ordinary epithelial cells. On the other hand, Andreasen (1952) claims to have seen ample evidence of lymphocytic degeneration in the epidermis, and states that this is one of the disposal areas for the lymphocytes of the body.

Bone marrow. From a comparative study of the haemopoietic organs of certain amphibia and fishes Jordan and Speidel (1923) advanced the hypothesis that the lymphocytes of the blood to a large extent are filtered out in the red bone marrow to function as progenitors of blood cells, both red and white. This hypothesis was supported, but

not proved, by Kindred (1942) in a quantitative study of the haemopoietic organs of rats. Kindred concluded from the mitosis figures that the production of lymphocytes can provide the necessary support which the erythroid population of the bone marrow requires from some outside source in order to produce sufficient red blood corpuscles to meet the demands of the blood. The method used by Kindred was criticized by Andreasen and Christensen (1949).

Yoffey and Parnell (1944) calculated the number of lymphocytes per cu.mm in the rabbit bone marrow, and found a figure considerably higher than the blood lymphocyte level. They calculated that the total lymphocyte count of bone marrow was of the same order of magnitude as the number estimated to be daily leaving the blood. Analogous conditions were found in guinea-pigs (Yoffey and Metcalf 1949, quoted by Yoffey 1950). Yoffey (1950) discusses whether the bone marrow lymphocytes are 'myelopetal or myelofugal, i.e. whether they are passing to the marrow from the blood, or in the reverse direction'. He argues that 'the nature of the lymphoid nodules found in bone marrow strongly suggests that the lymphocytes are myelopetal'. On the other hand, he emphasizes that 'Unfortunately, the identity of the lymphocyte has been the subject of controversy on so many occasions that any data appertaining to it have become suspect. An excellent instance is afforded by the bone marrow of the rat, in which the lymphocyte percentage has variously been estimated as 5.0 % (Stasney and Higgins, 1935), 20 % (Overbeck and Querido, 1938), 2.96 % (Higgins and Machella, 1939), 21.7 % (Aschkenasy, 1946) and 13.3 $\pm$ 4.27 % (Endicott and Ott, 1945)'. Spectrographical differences have been observed by Metcalf (1953) between blood lymphocytes of guinea-pigs and the bone marrow cells called lymphocytes by Yoffey. This does not, of course, prove that a real difference exists between the supposed lymphocytes in the bone marrow and the blood lymphocytes (cf. page 14). An indisputable, objective method of identifying the lymphocyte must, however, be developed before conclusions can be drawn from morphological studies of the above kind.

Bone. The most extravagant of all theories on the fate of the lymphocyte is that of Ökland (1940). He believes, on the basis of histological studies, that the lymphocytes are transformed into bone cells.

Connective tissue. It is most probable that a small number of lymphocytes are continuously escaping from the blood capillaries all

over the body into the connective tissue, thence finding their way into lymphatic capillaries (Clark and Clark 1930 and 1932, Yoffey and Drinker 1939). No quantitative studies of connective tissue lymphocytes have been made, as far as I know.

HISTOPATHOLOGICAL STUDIES. *Histopathology of inflammation.* The role of the blood-lymphocyte in the histopathology of inflammation has given rise to much conflict of opinion. A review of the literature is given by Kolouch (1939), and our knowledge has not increased much since then. The critical remarks in page 11 can be applied to most of the experimental approaches to this problem. The conclusive proof of observing the migration of an individual lymphocyte from the blood to the seat of inflammation, and observing its transformation into a macrophage, has yet to be made.

Bone marrow in conditions of maturation arrest. Drinker and Yoffey (1941) say that if it is true that many lymphocytes are constantly leaving the blood to enter the marrow, there to develop into granulocytes and erythrocytes, it should be possible to predict the accumulation of small lymphocytes in the marrow in conditions of maturation arrest. Summing up the marrow changes in various conditions of maturation arrest, they state that 'in agranulocytosis and certain of the aplastic anaemias quite definitely, and to a lesser degree in pernicious anaemia, lymphocytes appear in the bone marrow in increased numbers'. And '. . . it seems indisputable that lymphocytes can accumulate in the marrow in considerable numbers without any evidence of multiplication there'. Drinker and Yoffey themselves remark that cells called lymphocytes by them in these pathological conditions are called primitive cells and lymphoid cells by others (Rhoads and Miller 1938, Cohnheim 1876, both quoted by Drinker and Yoffey 1941). Besides the fact that these bone marrow changes can be explained in other ways, the drawing of analogies between pathological and normal conditions is very unreliable.

EXTIRPATION OF THE ORGAN SUPPOSED TO ELIMINATE OR TAKE UP LYMPHOCYTES. *Intestinal epithelium.* Erf (1940) removed the stomach and intestines in rabbits, and counted the number of lymphocytes in the blood. He found that 'viable lymphocytes, either autogenic

or heterogenic, administered intravenously to either normal or gastro-enterectomized rabbits, disappear very rapidly from the circulating blood. Excretion of lymphocytes through the mucosa of the stomach and intestines is not the only mechanism responsible for lymphocyte destruction'. A similar experiment was done by Yoffey (1942), who removed the major portion of the intestine, and found no constant change in the blood lymphocyte count. Both these experiments are very drastic, and because of the complex control of the level of the blood lymphocytes, no conclusions can be drawn.

The bone marrow. Steinberg and Martin (1946) describe a technique for removing the marrow from the long bones of rabbits and replacing it with inert material. In six animals in which they rapidly removed 41 $\pm$ 5 % of the total marrow the blood lymphocytes increased, reaching a maximum, 3 times the normal level, on the 5th day. The same objection can be raised against this experiment: it is too drastic, and no controls are available.

TISSUE CULTURE. Many investigators believe that they have proved the transformation of the small lymphocyte into different kinds of cells *in vitro*. From these tissue culture studies they have drawn conclusions about the disappearance of the lymphocyte from the blood. Thus the transformation of lymphocytes into typical macrophages has been demonstrated, and from this it has been concluded that the macrophages of inflammation are partly derived from blood lymphocytes (see page 11). It has also been shown that lymphocytes *in vitro* can become myelocytes (Maximow 1923) and granulocytes (Bloom 1937), and a filtering-out process of the lymphocytes in the bone marrow has been inferred.

Tissue culture as a method in these connections has been criticized on page 11. It is possible that conclusions about the lymphocytes based on tissue culture studies apply to a small number of the lymphocytes, the youngest of them, but it seems improbable that they are pertinent to the majority. The same opinion is expressed by Mas y Magro (1948), discussing 'follicular lymphocytes', which he considers to be undifferentiated cells, and 'functional lymphocytes', which he believes to be the fully developed cells.

TRANSFUSION OF LYMPHOCYTES. Erf (1940), and Hammond and Novak (1950), who counted the number of blood lymphocytes after intravenous injection of large amounts of these cells, state that the injected cells circulate for a period of about 30 minutes only. They used different methods, Hammond and Novak criticizing Erf. No attempts were made to trace the injected cells. All conclusions based on blood counts must be regarded as very uncertain. Osogoe (1950) made an investigation using homoiotransfusion of lymphocytes. A suspension of cellular constituents from the mesenteric lymph nodes of rabbits was given in a single injection, either into the ear vein or mesenteric vein of another healthy rabbit. From the 48th to the 72nd hour thereafter there occurred a striking focal accumulation of lymphocytes, including large lymphocytes and lymphoblasts, in the radicles of the interlobular portal vein, in the periportal spaces of the liver, and in the perifollicular regions of the spleen. The accumulations of lymphocytes in the spleen occurred somewhat later than those in the liver, reaching the maximum at the 72nd hour. When the cellular elements of the suspension were killed or severely injured before injection no accumulations of lymphocytes were produced anywhere. A similar experiment was performed in which large quantities of lymphocytes from thymus were transfused (Osogoe and Hitachi 1950). As previously mentioned, page 15, the result was almost the same. The weak point of Osogoe's method is that the cells are not labelled at all, and that the lymphocytes are shown in histological sections without the possibility of a certain, quantitative comparison.

Farr (1951) made autotransfusions of labelled lymphocytes in rabbits. The lymphocytes were obtained from the popliteal lymph nodes, and their nuclei labelled with 3,6,diamino-10-methylacridinium chloride. The rabbits were killed 2 and 12 hours after the injection. After 2 hours, lymphocytes with labelled nuclei were found in the bone marrow and lymphoid tissue, including the thymus, mesenteric lymph nodes, appendix, Peyer's patches, and the white pulp of the spleen. At 12 hours labelled lymphocytes were still present in lymphoid tissue, but at this time most of the labelled lymphocytes in bone marrow had apparently become myelocytes. No labelled cells of any type were seen in the tissues of rabbits either 2 or 12 hours after the injection of labelled guinea-pig lymphocytes. Concerning Farr's method it may be mentioned that the injected cells are shown in histological sections, and quantitative comparisons are

thus very uncertain. The labelling of the lymphocytes with stain is a haphazard procedure.

Large numbers of lymphocytes labelled with P^{32}, obtained from the intestinal lymphatics, were transfused from rat to rat (Weisberger, Guyton, Heinle, and Storaasli 1951). 10—15 minutes after the intravenous or intra-arterial injection the greater part of the radioactivity was recovered from the tissues of the lungs, liver, spleen, and kidneys. Of these organs the lungs and the spleen were radioactive to a degree far in excess of that found in animals which received comparable intravenous doses of inorganic P^{32}. Since the receptors were not examined later than after 10—15 minutes the authors cannot say whether the lymphocytes were definitely removed from the circulation or not. Osogoe (1950), who noticed independently the same accumulation of transfused lymphocytes in the lungs, writes, 'The majority of the lymphocytes injected into the ear vein are at first arrested by the lung capillaries as a result of mechanical regulation by these capillaries. Similarly, lymphocytes injected into the mesenteric vein by the intralobular capillary sinusoids of the liver'. And Osogoe continues, 'In the further behavior of the injected lymphocytes, it is important that a period of 24 to 48 hours must elapse before a considerable accumulation of lymphocytes occurs in the particular area as of the liver and spleen, even when suspensions are injected directly into the portal circulation. This fact suggests that the process is no longer mechanical, but characteristic of the lymphocyte'. The following experiment by Ambeus *et al.* (1953) gives support to the view that transfused lymphocytes are mechanically accumulated in the capillaries of the lungs. The fate of white blood cells was studied in Starling's heart-lung preparation. The lungs seemed to remove white blood cells from the circulation until a specific level was reached, which then was maintained. If white-cell-free blood was introduced into the preparation a slow release of white cells occurred from the lungs. A temporary accumulation of white blood cells in the lungs was also demonstrated by Bierman *et al.* (1951) who studied the effect of intravenous histamine administration on the level of white blood count in the peripheral blood.

Before these recent transfusion experiments were published transfusion of P^{32} labelled lymphocytes was started here. A preliminary report of these experiments has already been published (Fichtelius 1952).

CHAPTER IV

RADIOACTIVE PHOSPHORUS

PHYSICAL AND BIOLOGICAL DATA OF P^{32}

The data given in this chapter are, except where mentioned, taken from Kamen, Radioactive Tracers in Biology, 1951, and Hevesy, Radioactive Indicators, 1948.

Radioactive phosphorus, P^{32}, is a β-particle-emitter, the upper energy limit being 1.69 MEV. Although P^{32} nuclei do not emit γ rays, the presence of Bremsstrahlung can be observed when strong P^{32} samples are employed. The Bremsstrahlung, given off by β rays from P^{32}, amounts to 1 γ quantum per 25 β particles when the retardation takes place in lead. The half-life is 14.3 days. Self-absorption corrections do not begin to be appreciable until thicknesses of over 50 mg/cm^2 are encountered. The half-life is long enough to render decay corrections unnecessary when samples are assayed over a period of less than 12 hours. The isotope was produced in the pile at Harwell, England. Recently evidence has been accumulated of the presence of small amounts of the isotope P^{33} in samples of P^{32} manufactured from atomic piles (Westermark 1951, quoted by Odeblad 1952). This isotope has a half-life of about 25 days, and a maximum β energy of about 0.26 MEV. A small amount of P^{33} seems to be present in the solution used here, but it has not affected the investigations described in this paper. The P^{32} was obtained as carrier-free H_3PO_4 containing about 3—10 mC per cc. After dilution with normal saline the solution, now containing 10—500 μC per cc., was sterilized, and thus was ready for administration.

After subcutaneous and intravenous injection of P^{32}, the routes of administration used here, the isotope does not remain for very long in the blood plasma. According to Hevesy and Ottesen (1943) the percentage of the subcutaneously administered P^{32} present in 1 mg inorganic P from rat plasma, at different times after injection are:

2 hrs.	5 hrs.	8.5 hrs.	13 hrs.	25 hrs.	50 hrs.	72 hrs.	94 hrs.
0.91	0.67	0.214	0.286	0.204	0.114	0.099	0.069

The activity of inorganic P^{32} in rabbit plasma falls to 50 per cent of the maximum activity within 2 hours of subcutaneous injection (Hevesy and Hahn 1940). In the above example the activity after 4 days is estimated to be only 4 per cent of the maximum activity.

Plasma phosphate is in rapid equilibrium with the phosphate of various organs. The diffusion of phosphate from blood plasma into the extracellular space alone accounts for a nearly eight-fold dilution. On the dynamic state of body constituents Hevesy (1948) writes, 'A phosphate radical taken up with the food may first participate in the phosphorylation of glucose in the intestinal mucosa, soon afterwards pass into the circulation as inorganic phosphate, enter a red corpuscle, become incorporated with an adenosinetriphosphoric acid molecule, participate in a glucolytic process going on in the corpuscle, return to the circulation, penetrate to liver cells, participate in the formation of a phosphatide molecule, and after a short interval enter the circulation in this form, penetrate marrow, and leave this organ after some time as a constituent of a lymphocyte. We may meet the phosphate radical again as a constituent of the plasma, from which it may find its way into the skeleton. Being incorporated in the uppermost molecular layer of the skeleton it will have a good chance of being replaced by other phosphate radicals of the plasma or the lymph, but it may also have the good fortune to find a more or less lasting abode in the skeleton'.

Parenterally administered P^{32} is excreted through the kidneys and bowels. The following figures (taken from Hevesy 1948, Gaunt, Griffith, and Irving 1942) refer to subcutaneous injection in rats.

Percentage of P^{32} excreted

	after 24 hours	after 90 hours
Urine . . .	8.5	24.3
Faeces . . .	0.23	2.6

Intracellular formation of phosphate-containing molecules requires penetration of plasma inorganic phosphate into the cell. The appearance of phosphate within cells depends primarily on the metabolic activity of the cells. The metabolism of phosphorus is linked with protein metabolism by way of nucleic acids and nucleoproteins. Desoxyribose nucleic acid (DNA) is wholly or largely confined to cell nuclei, and the DNA content of the nuclei increases and decreases in the various stages of mitosis. Thus there is an appreciable

turnover of DNA in growing tissue and in organs, such as thymus, spleen, and bone marrow, which secrete products containing DNA. That the rapid accumulation of P^{32} in nuclei of growing tissue may be attributed to mitotic activity is suggested by an experiment carried out by Marshak (1941).

THE CHOICE OF P^{32} FOR LABELLING LYMPHOCYTES

The choice of P^{32} as an indicator was, in addition to some practical considerations, essentially based on three facts: 1. The P^{32} incorporated into DNA molecules during the formation of white blood corpuscles remains in the cells throughout their lifetime. Hevesy and Ottesen (1945) and Ottesen (1948) have shown that the nucleated red cells in hens retain their DNA intact until the cells degenerate. Ottesen (1948) has also shown that the same applies to the white blood corpuscles of the hen. Further support to this view has been given by Kline and Cliffton (1952), in an investigation on the life-span of human leucocytes; 2. There is a very intense turnover of DNA in lymphocyte-producing organs (Andreasen and Ottesen 1944) and P^{32} is built into the DNA of lymphocytes to such a large extent that it is not only possible to trace them in the body of the animal (Ottesen 1948) but also to obtain a lymphocyte suspension of such high activity that the cells can be followed even when introduced into a second animal; 3. Shemin (1948) has shown that P^{32} contained in the debris of degenerated red cells of the hen is not preferentially used in the formation of new cells. There is no reason to believe that P^{32} of destroyed lymphocytes would differ in this respect.

RADIOSENSITIVITY OF LYMPHOCYTES

Lymphocytes are generally regarded as the most radiosensitive cells in the body. A drop in the lymphocyte count is the earliest, most marked, and most consistent, blood change induced by therapeutic radiation (Minot and Spurling 1924). The same change can be induced by internally deposited radio-isotopes (Jakobson and Marks 1947). The lymphocyte count, however, depends on many unknown factors, and gives questionable information. Warren, McMillan, and Dixon presented in 1950 a histological survey of the effects of internal radiation of mice with varying doses of P^{32}, of which the smallest (0.80 μC/g body-weight) was comparable to the

dose used in my transfusion experiments. The thymus glands of the mice which received this dose increased in size ten days after the injection, and lymphopoietic hyperplasia was present throughout the medulla and cortex. This hyperplasia can be looked upon as compensatory to an initial decrease in lymphopoiesis. Such a decrease was not demonstrated after 0.80 μC/g, but that it occurred seems probable from experiments using larger doses (Warren and Dixon 1949, Warren, McMillan, and Dixon 1950).

It may be said that some of the responses to radiation are similar to those occurring in other forms of stress (Edelman 1948, Ellinger 1948, Prosser 1948, all quoted by Edelmann 1951). The drop in the lymphocyte counts and the histological changes in the thymus may be explained in this way. The lymphocyte-producing organs may be very radiosensitive via the adrenals, but the lymphocytes themselves may be as resistant as other cells, and labelled lymphocytes may behave in the same way as unlabelled. It cannot, however, be denied that lymphocytes formed under the influence of ionizing radiation are morphologically altered. For instance a study of supravital preparations of blood cells from persons exposed to radiation indicated a striking increase in the number of refractive neutral red bodies in the cytoplasm of the circulating lymphocytes (Dickie and Hempelmann 1947). The labelling of lymphocytes with radioactive isotopes must be fraught with chance until the labelled cells have been shown to behave like unlabelled *in vivo*.

CHAPTER V

METHODS

ISOLATION AND WET ASHING OF DNA

DNA was isolated from different organs by a method described by Schmidt and Thannhauser (1945). The acid-soluble and lipid-P was removed from the finely divided organ by treatment with trichloracetic acid, water, alcohol, ether, methanol, and chloroform. The tissue powder was then incubated with potassium hydroxide until completely dissolved. The DNA was precipitated by addition of a mixture of hydrochloric acid and trichloracetic acid. Schmidt and Thannhauser determined the DNAP as the difference between the total phosphorus of the alkaline hydrolysate and the total phosphorus of the acid supernatant. Their method was modified as follows, to accord with the subsequent determination of radioactivity. The precipitated DNA was collected on a small filter-paper (ashed weight 0.1 mg), which was then allowed to dry in air for 24 hours before wet ashing. The wet ashing of the DNA was performed according to directions given by Teorell (1931). The remainder was cooled, diluted with distilled water, and boiled for a few minutes as recommended by Andreasen (1943). Subsequently one of the following alternatives was followed. 1. The sulphuric acid solution was transferred into a 25 cc. volumetric flask and made up to the mark with distilled water. Of this solution 5 cc. was taken for determination of phosphorus and 20 cc. for determination of radioactivity (experiments 1, 2, 3, and 8). 2. The solution was transferred into a 10 cc. volumetric flask and made up to the mark. 4 cc. was used for P-determination and 6 cc. for activity-determination (experiments 4, 5, 6, and 7).

DETERMINATION OF PHOSPHORUS

The method employed was worked out in principle by Fiske and Subbarow (1925), and modified by Teorell (1931). The principles are as follows. P, which is present as PO_4, is transformed into phos-

phomolybdic acid, which is then reduced by means of a reducing substance to a compound which turns blue in the presence of sulphite. The extinction of the resulting blue solution is determined in a spectrophotometer, and the phosphorus concentration calculated from a calibration curve.

The preparation of samples for measurement of extinction was carried out according to directions given by Andreasen (1943), except that the reducing substance recommended by Teorell (1931) was used. Details are given to preclude uncertainty: as before there were two alternative procedures. 1. 5 cc. of the specimen (the P-concentration of which is to be determined) were transferred into a 25 cc. volumetric flask. The sulphuric acid solution was neutralized by addition of 5N—NaOH to weak alkaline reaction, and then acidified slightly by means of 1N—HCl. 5 cc. of 5N—H_2SO_4, containing 2.5 per cent ammonium molybdate, was then added, and the flask made up to the mark with distilled water. Finally 1 cc. of reducing fluid (Teorell) was added, and the sample allowed to stand for 15 minutes. The extinctions were read during the following 30 minutes. 2. 4 cc. of the specimen was transferred into a 10 cc. volumetric flask, and neutralized and acidified as above. 2 cc. of the mixture of H_2SO_4 and ammonium molybdate, and 0.4 cc. of the reducing fluid, were added, and the solution made up to the mark. Subsequently as 1.

The same type of filter-paper which was employed for the separation of the DNA was used in preparing the blank. It was treated exactly as the samples.

The quantitative absorption measurements were performed in the Beckman Model DU Spectrophotometer at 720 millimicrons. In making these measurements the concentration of the solution and the light-path length of the cell must be taken into account. The extinction coefficient (k) was calculated from measurements made on solutions of known concentration, as follows:

$$E = k \times c \times l, \text{ where}$$

$$E = \log_{10}\left(\frac{I_0}{I}\right)$$

c = concentration of sample in solution

l = light-path of the cell

Beer-Lambert's law did not, however, hold for the lowest concentrations, and it was necessary to plot a calibration curve of E

vs. mg P in the sample. The quantitative determination was then made by applying the observed value of E to this curve. The calibration curves were based on analyses of known, varying amounts of phosphorus as secondary sodium phosphate in 0.01 N—HCl. The specimens were prepared for assay in 25 cc. and 10 cc. volumetric flasks as described in page 27, except that it was not necessary to neutralize the starting solution. E was calculated for 5 and 6 known quantities of phosphorus from 5×11 and 6×11 single determinations in the 25 cc. and 10 cc. flasks respectively. Corrections were made for the different light-paths and transmissions of the individual cells. Different stock solutions of P were used, and the standard deviations of E were thus larger than if a single stock solution had been employed throughout the test. On the other hand, the relationship between the obtained and the true curves is more likely to be closer if several stock solutions are used than if the curves are based on only one. The calibration curves and the standard deviations of E are shown in Fig. 1. The complexity of the standard deviations are indicated by oblique lines.

DETERMINATION OF RADIOACTIVITY

The starting material for the determination of radioactivity was the acid solution of orthophosphate obtained after wet ashing. The preparation of specimens was performed by the method of Lindberg (1946). The principles only are quoted here. 'As the orthophosphate in the sample is generally too little to be precipitated quantitatively, a known amount of Na_2HPO_4 is added so that the amount will always be the same in the sample. All the orthophosphate is then precipitated as $MgNH_4PO_4$'. In this way the self-absorption was of the same magnitude in all specimens, and the quantities remaining in the solution were so small in proportion to the precipitate that they could be neglected. The standard preparation used for elimination of the disturbing effect of decay (see page 30) was made in a less time-consuming way. An aliquot, for instance 0.025 cc. of the solution administered to the animals, was diluted, say, 25 000 times. Of this highly diluted solution 0.1 cc. was transferred to a non-perforated aluminium cup in which a small filter-paper was placed. After evaporation to dryness at 75°C the filter-paper was secured with a few drops of shellac. In this preparation too the self-absorp-

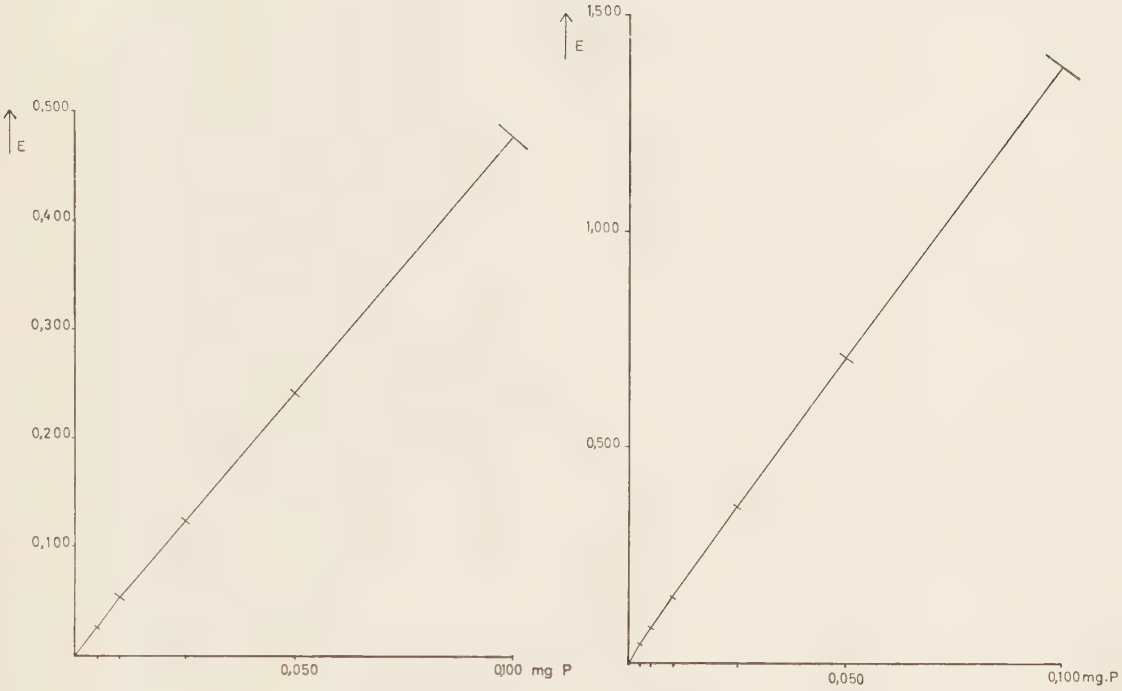

Fig. 1. Calibration curves for 25 and 10 cc. flasks respectively. The standard deviations of E are indicated.

tion was neglected, because the amount of substance was equally small in every case.

The radioactivity was measured with self-quenching, bell-type, Geiger-Müller tubes, three types of which were employed in the course of the investigations. These were EW3H, from The 20th Century Electronics Ltd., London; LKB 5007, from Aktiebolaget LKB-produkter, Stockholm, Sweden; and M1, from Brüel and Kjaer, Copenhagen, Denmark.

The electronic scaler, type number 6501, manufactured by Brüel and Kjaer, Copenhagen, has the following qualities: the resolving time of the amplifier is about $5 \cdot 10^{-5}$ sec.; the dividing scale can be extended to 64; 'the high tension is stabilized so that a variation of 5 % of the line voltage gives a variation of less than 0.1 % in the high tension'.

The radioactivity of each specimen was registered once. As a rule more than 2 500, and in 9 % less than 2 000, impulses were counted.

The number of impulses/minute was usually less than 1 000, and 4 % of all measurements exceeded 2 000. In 15 % the assayed activity was lower than the background counting rate. As the counting rates were so low no corrections for the resolving time of the counter were necessary. The background counting rate was evaluated during more than 1 hour on each day on which measurements were made. Correction for decay-rate was avoided by comparing the activity of the sample to be determined with the activity of an aliquot of the preparation administered. No correction was necessary for the different effectivities of the tubes used: in all experiments two or more specimens were compared, and these were always assayed with the same tube. The condition of the equipment was checked frequently by plotting the characteristic curves of the tubes, using an uranium standard. In addition the tubes were checked every day on which measurements were performed, by measuring the activity of this uranium-standard. The function and sensitivity of the electronic scaler were verified by means of an impulse generator (type 6501b, Brüel and Kjaer). Painstaking attention was devoted to the cleaning of used pipettes, test tubes, and other glassware, and these were frequently checked with a Geiger-Müller Monitor after cleaning.

ERRORS OF ESTIMATION

To check the reproducibility of the phosphorus determination, double and quadruple measurements were performed on DNAP isolated from liver. After wet ashing the samples were diluted to 25 cc. and 10 cc. (see page 27). Twelve quadruple determinations were done in the case of the 25 cc. flasks; 18 double determinations were done from the 10 cc. flasks. The standard deviation, σ, of a single determination was calculated according to the following formula:

$$\sigma^2 = \frac{\sum_{i=1}^{n} \left(x_i - \bar{x}\right)^2}{n - 1}, \text{ where}$$

$x_i, x_{i+1}, \ldots . x_n =$ the single observations,
$n =$ the number of observations, and
$\bar{x} =$ the arithmetical mean.

For 25 cc. flasks $\frac{\sigma}{\bar{x}}$ was found to be 1.2 % in this test, and for the whole investigation 0.6 %. For 10 cc. flasks $\frac{\sigma}{\bar{x}}$ was 0.6 % in the test, and 2.3 % for the whole investigation.

The error of the method of determining P^{32} has two major components — the error depending on the random decay of the isotope, and that connected with the preparation of the sample. The standard deviation in counts accociated with the total number of counts in a given period is given by the equation

$$E_n = \sqrt{N}, \text{ where } N = \text{the total number of counts.}$$

When measuring 2 500 individual counts there is a 2/3 probability that the activity measured is within ± 2 % of the true activity (viz. the activity which would result from measurement over an infinite period). For strong samples the standard deviation can be computed with sufficient accuracy according to the above formula. For weak samples the background rate contributes appreciably to the statistical error. The combined error is the square root of the sums of the squares of the individual errors. It can be calculated that, under the conditions described on page 29, the standard deviation of the result is about 4—5 % when the activity of the sample is equal to the background. The error connected with the preparation of the sample has not been calculated.

The total error connected with the determination of radioactivity per mg DNAP depends on the errors of the determinations of radioactivity and of DNAP. An estimation of this total error was performed as a part of experiment 3. 28 double determinations were performed, and in 15 of these the activity of the sample was lower than the background counting rate. Thus the error of the determination of radioactivity is very large in this test in comparison with the error of the rest of the investigation (see page 30). The standard deviation of the determination of DNAP in the test was 1.3 % of the mean of the observations, i.e. larger than that valid for the largest part of the investigation, where $\frac{\sigma}{\bar{x}} = 0.6$ %. For the determination of the activity per mg. DNAP in this test $\sigma = 9.6$ %. From this it is clear that the standard deviation of the single determination of radioactivity per mg. DNAP in the rest of the investigation is usually less than 9.6 % of the observation.

Hevesy (1948) emphasizes the utmost importance of purifying the sample of DNA very carefully in order to remove phosphorus compounds that may have a much higher spec. act. This applies to shorter experiments in which the spec. act. of inorganic P is very high. In these investigations the experiments were never shorter

than 24 hours, and thus it was of less importance to purify the DNA. The error in the determination of the radioactivity per mg. DNAP includes the error due to impurities.

In experiments 4—7 the activity of each DNAP-sample was compared with the sum of the activity of two standard preparations. 16 determinations of such pairs were performed, and the standard deviation of a single observation was 0.03 × the observation. The standard deviation of the sum of a pair of observations was thus $\frac{0.03}{\sqrt{2}}$ × the sum.

CHAPTER VI

TRANSFUSION OF LABELLED LYMPHOCYTES

HETERO- AND SYNGENESIO-TRANSFUSION

The theory of acquired immunity as the cause of biological incompatibility has within recent years gained increasing ground, but it is not accepted everywhere. The reader is referred to a critical review of the literature given by Simonsen (1953). Only the most important of the investigations on which this theory is based are mentioned here.

Medawar *et al.* (1948, quoted by Simonsen 1953) showed 'that homografted skin, after a latency during which the homograft was indistinguishable from an autograft, always was destroyed, and nearly always within one or two weeks. The recipient had now changed, so that a secondary skin graft from the same donor was destroyed more quickly than the primary, whereas a succeeding graft from another donor usually suffered less damage than the secondary graft from the first donor. The altered response of the recipient was not only local, but comprised the entire skin area (and other parts of the organism) — — —. Although Medawar was unable to demonstrate the operative antibodies *in vitro*, his results are actually explainable only by acquired immunity, unless we introduce the purely hypothetical concept of a defence mechanism, characterized by following in part the same general laws as antibody formation, but otherwise indefinable. — — —. Unfortunately nobody has ever properly investigated when transplantation immunity begins, when it reaches its maximum, and how long it lasts. This is probably in part due to the extraordinary difficulties associated with *in vitro* determination of the active antibodies. If we take the histological changes in the transplant as a sign that immunity processes are developing, as we shall not hesitate to do, we may say that the antibody production is well at work already after 3 or 4 days. This is, indeed, earlier than antibodies are usually demonstrable, unless it is a question of secondary response. Simonsen and Christensen (1950) found, however, by parabiosis of rats with mice that

both parabionts regularly formed antibodies against each other's blood cells after 3 or 4 days'.

In this investigation radioactively labelled lymphocytes were transfused, 1. from rabbit to rat — heterotransfusion — and 2. from rat to rat within the same litter, the animals having been inbred for several years — syngenesiotransfusion.

From a biological point of view it is possible that both the syngenesio- and the hetero-transfused lymphocytes remained alive and behaved as the lymphocytes of the receptor during the 24 hours of my experiments. This inference is substantiated by Wesslén (1952). His findings made it seem likely that the lymphocytes in the form in which they enter the blood stream do not contain any antibodies, but are capable of producing them. Guinea-pigs that received viable thoracic duct lymphocytes from sensitized rabbits reacted with severe anaphylactic shock upon intravenous antigen injection, whereas those receiving the same amount of lysed lymphocytes from the same animal were quite unaffected by the antigen injection. Similar results were obtained when immune-horse lymphocytes were transferred to normal guinea-pigs. These findings indicate that the lymphocytes, in spite of heterologous transfer, can be viable in the new host.

It must be emphasized that, even though injected lymphocytes of both kinds do behave as the lymphocytes of the receptor, syngenesio- and hetero-transfused cells do not necessarily behave in the same way. The lymphocytes of an individual animal may perhaps function in different ways under different conditions, and those different conditions may obtain with regard to the lymphocytes themselves as well as to the rest of the organism. In syngenesiotransfusion the donors and the receptor have acquired immunity against the same infections, but this is not the case in heterotransfusion. Thus the conditions applying to the two forms of lymphocytes are different, and accordingly they may behave differently.

The syngenesiotransfused cells contain individual-specific antigen that gives rise to a relatively small production of individual-specific antibodies (see page 5). The heterotransfused lymphocytes contain individual-specific and species-specific antigens, and give rise to stronger antibody production (see page 5). On transfusion of lymphocytes antigen is thus administered at the same time, to a lesser degree in syngenesiotransfusion than in heterotransfusion. It has been shown that antigens are taken up predominantly by reticulo-

endothelial cells throughout the body (Coons 1953, McMaster 1953). Because of this the injected antigen, viz. the lymphocytes, may be taken up by the RES of the receptor.

There would thus appear to be three possible fates of the transfused lymphocytes, examined after 24 hours. 1. They may be dissolved (perhaps after a short stay in the RES). The $DNAP^{32}$ thus set free would be distributed throughout the body (see page 23). 2. They may be taken up as antigens by the RES, and in smaller concentrations elsewhere too. 3. They may behave like the lymphocytes of the receptor, either after both transfusions or after syngenesiotransfusion alone.

PREPARATION OF A SUSPENSION OF RADIOACTIVE LYMPHOCYTES

Thymus was used as the starting material. In the case of syngenesiotransfusion thymus was taken from 2 or 3 rats, 3 months old: in heterotransfusion thymus from 1 rabbit weighing about 0.6 kg. was used. The gland was cut into about 10 pieces. By forcibly injecting 0.9 % sodium chloride into each of the pieces and cautiously squeezing with forceps a suspension of lymphocytes was obtained. The pieces were then removed, and the debris allowed to sediment for 1 minute. The supernatant fluid was then drawn into a syringe and was ready for injection. No attempt was made to wash the lymphocytes, in order to avoid injuring the cells unnecessarily. The lymphocytes were kept *in vitro* for 15—20 minutes in all, and the whole procedure was carried out under sterile conditions. The suspension, altogether about 1 cc., contained in the case of syngenesiotransfusion an average of 340 000 cells per cu.mm: in heterotransfusion the corresponding number was 300 000. Some differential counts were performed, which revealed almost exclusively lymphocytes, 1—2 % being granulocytes.

Radioactive phosphorus is incorporated into DNA molecules of the lymphocytes during the formation of the cells (see page 23). The simplest way of obtaining a suspension of labelled lymphocytes is to label a whole animal and then to prepare a suspension of lymphocytes from the largest producer of these cells, the thymus. The injected cells should be as strongly labelled as possible, to facilitate the demonstration of P^{32} in the DNA of the transfused cells within the recipient. Two questions now arise. 1. What time should elapse be-

tween the injection of P^{32} and the killing of the donor? Is it worth the trouble of maintaining a constant concentration of inorganic P^{32} in the tissue fluid by repeated injections? 2. What is the largest dose of P^{32} which can be injected, bearing in mind the disturbing effect of radiation on lymphopoiesis (see page 24)?

It has been shown (Fichtelius 1951) that on the 3rd day after the injection of P^{32} the number of radioactive lymphocytes in the blood is near its maximum. Supposing that at least some of these cells are derived from the thymus, we can say that the time allowed to elapse between the injection of P^{32} and the preparation of the lymphocyte suspension — the 'labelling time', — must not exceed 2 days. After that time the most radioactive of the lymphocytes are probably leaving the thymus. Further information can be obtained from an experiment by Andreasen and Ottesen (1944). With the aid of P^{32} they estimated the percentage renewal of DNA in thymus in 24 hours. This value was calculated from two experiments lasting 2 hours and 42 hours respectively. If all cells formed during the experiment were to be found in the organ at the end of the experiment, identical values for the percentage renewal of DNA in 24 hours could be expected. This was not, however, the finding. The value from the longer experiment was 31.8, and that from the shorter experiment 41.0. This may be because some of the cells formed during the experiment had left the thymus 42 hours after the injection of P^{32}. The choice of the labelling time used in these investigations — 24 hours — was influenced by the following experiment, carried out in 3-month-old rats. 4 μC P^{32} was injected subcutaneously, the thymus excised after different times, and a suspension of lymphocytes prepared. The suspension was washed once in normal saline by centrifugation. (Further washings were shown to give a very small exchange of inorganic P^{32}.) The spec. act. of the lymphocyte suspension was calculated as the number of impulses per 100×10^6 lymphocytes per cent of the number of impulses injected. It was shown that the spec. act. determined in this way was highest after a labelling time of 24 hours. The method used is very crude, and charged with great inaccuracies, but gives, nevertheless, some additional information. With regard to the above findings it is considered unnecessary to maintain a constant concentration of inorganic P^{32} in tissue fluid.

The effect of ionizing-radiation on different types of tissue has been studied by Hevesy (1945): he determined the amount of newly

formed DNA before and after irradiation, using P^{32} as an indicator. The same principle has been used in an attempt to estimate the optimum dose of P^{32} administered. Between 5 and 250 μC was injected into 3-month-old rats, the thymus excised after 17 hours, and a suspension of lymphocytes prepared. This suspension was washed once in normal saline by centrifuging, as in the previous experiment, and the spec. act. determined in the same way — viz. the number of impulses per 100×10^6 lymphocytes per cent of the number of impulses injected. The spec. act. is a crude measure of the total phosphorus turnover, including the turnover of DNAP. It was supposed that identical values for the spec. act. would be obtained with different doses of P^{32} provided that lymphopoiesis was not reduced by radiation. No change in the spec. act. was noticed, even with the largest dose (250 μC). It was calculated that 250 μC given to a 3-month-old rat yielded a lymphocyte suspension of about 0.3 μC 24 hours after injection. The corresponding figure for 150 μC was 0.2 μC. Using 3 rats as donors the amount of P^{32} in the obtained lymphocyte suspension was large enough to be demonstrable as $DNAP^{32}$ in the receptor, even when 150 μC was used as the labelling dose. In these investigations still smaller doses were used (0.75 μC/g body-weight).

In order to check the method used in the experiments described above, the susceptibility of labelled lymphocytes *in vitro* was examined. 150 μC was injected into each of two rats, 3 months old. The animals were killed after 17 hours, and a lymphocyte suspension prepared from the thymus glands. After washing in normal saline by centrifuging, the number of impulses per 100×10^6 lymphocytes was measured. The suspensions were then washed twice more. During these manipulations the lymphocyte counts decreased to less than half the starting counts, but the number of impulses per 100×10^6 lymphocytes did not change appreciably. The suspension contains a mixture of cells: 1. cells formed during the period when the inorganic P^{32} in tissue fluid was maximum, the most radioactive cells; 2. cells formed immediately before the animals were killed (these contained less P^{32}); 3. cells formed before the injection, and thus without P^{32} in their DNA but with P^{32} in other P-containing fractions of the cells. If the most radioactive cells were more susceptible to the environmental influences of the experiment the number of impulses per 100×10^6 lymphocytes could be expected to fall after the washings. This is not the case, and the labelled cells are thus destroyed irrespectively of their content of P^{32}.

TRANSFUSION OF LABELLED LYMPHOCYTES IN NORMAL RATS

Experiment 1.

The first experiment on transfusion of labelled lymphocytes was based on the doubtful hypothesis that syngenesiotransfused cells did function in the receptor, but that heterotransfused cells did not. Heterotransfusions were thus intended to serve as controls, and the following question was set up: Is there any difference in the distribution of $DNAP^{32}$ among certain organs after syngenesio- and heterotransfusion of lymphocytes labelled with P^{32}?

Procedure. The experiment was divided into 2 series. In the first, lymphocytes from 2 or 3 inbred white rats of the same litter were injected into another rat of the same litter (syngenesiotransfusion). In the second, rabbit lymphocytes were transfused into rats (heterotransfusion). 18 syngenesio- and 20 hetero-transfusions were performed. In both series the suspension of labelled lymphocytes (see page 35) was injected into the femoral vein of 3-month-old rats weighing about 150 g. The injections were performed at the same time of the day. Immediately before the injection 0.8—1 cc. of blood was let from the tail of the receptor to reduce the load on the circulatory system. The injections were performed under ether anaesthesia, and lasted 3—5 minutes. After 24 hours the animals were killed, and the $DNAP^{32}$ from thymus, liver, the mucous membrane of the small intestine, lymph nodes, bone marrow, lungs, and spleen, was determined, and expressed in units of relative activity. By the relative activity of a certain organ is here meant the number of impulses/minute/mg. DNAP from the organ in question in % of the sum of the number of impulses /minute/mg. DNAP from the liver, mucous membrane of the small intestine, bone marrow, and spleen.

The result is evident from Fig. 2 and Table 2 (Chapter X).

The quotient S/H and its standard error were calculated for all organs. The critical ratio was then calculated: it is here defined as the difference between two quotients divided by the square root of the sum of the squares of the standard errors. The quotient S/H is the same in all organs except the spleen — viz. about 0.76. In the spleen $S/H = 2.17$, and there is a strongly significant difference be-

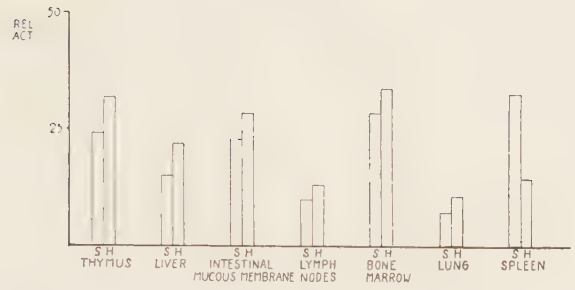

Fig. 2. Distribution of $DNAP^{32}$ after syngenesiotransfusion (S) and heterotransfusion (H) of radioactively labelled lymphocytes. The heights of the columns indicate the relative activity of DNAP (liver + mucous membrane of the small intestine + bone marrow + spleen = 100).

tween S/H of spleen and S/H of each of the other organs examined (the reference organs).

A certain part of the relative activity in the spleen after syngenesiotransfusion is supposed to be due to the transfused lymphocytes, and is estimated to be = S spleen — S/H reference organs × H spleen (see Fig. 3).

The question put on page 38 can thus be answered affirmatively, but of cause the hypothesis, that syngenesiotransfused cells function and heterotransfused do not, has not been prooved. It can be said that syngenesio- and hetero-transfused cells are not received in the same way.

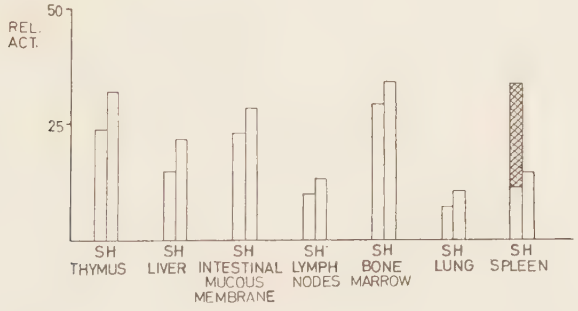

Fig. 3. Interpretation of the result of experiment 1. That part of the relative activity which is supposed to be due to syngenesiotransfused lymphocytes is shown cross-hatched.

DISTRIBUTION OF DNAP^{32} IN RATS AFTER ADMINISTRATION OF INORGANIC P^{32}

Experiment 2.

To facilitate the interpretation of the result of experiment 1 it is necessary to know the distribution of DNAP^{32} 24 hours after the injection into rats of inorganic P^{32}. The first problem of experiment 2 is whether there is any difference in the distribution of DNAP^{32} among certain organs after heterotransfusion of labelled lymphocytes, and after injection of inorganic P^{32}.

Procedure. The experiment was performed on 15 white rats, 3 months old and weighing about 130 g. At the beginning of the experiment 0.05 μC P^{32} per g body-weight was injected subcutaneously, and after 24 hours the animals were killed. The relative activity of DNAP from the mucous membrane of the small intestine, bone marrow, liver, and spleen, was calculated in the same way as in experiment 1.

The result can be read from Fig. 4, in which the corresponding activities after both transfusions are also shown. See also Table 3 (Chapter X).

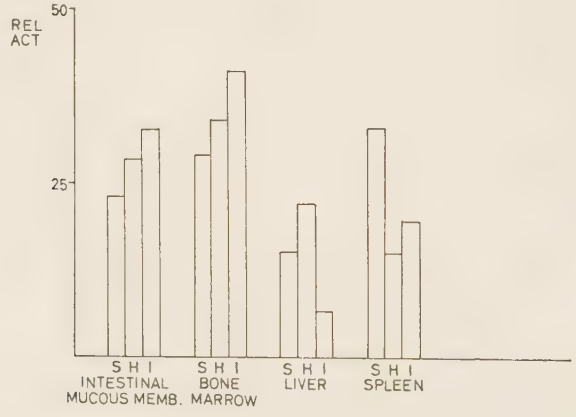

Fig. 4. Distribution of DNAP^{32} after injection of syngenesiotransfused lymphocytes (S), heterotransfused lymphocytes (H), and inorganic P^{32} (I). The heights of the columns indicate the relative activity (mucous membrane of the small intestine + bone marrow + liver + spleen = 100).

The quotient H/I is 0.87, 0.83, and 0.77, in small intestine, bone marrow, and spleen, respectively. H/I liver is 3.37, and there is a strongly significant difference between the H/I of the liver and the H/I of each of the reference organs (small intestine, bone marrow, and spleen). A certain part of the rel. act. in the liver after heterotransfusion is supposed to be due to the heterologous lymphocytes, and is estimated to be equal to H liver — H/I reference organs $\times$ I liver (see Fig. 5.). S/I small intestine and S/I bone marrow are the same (0.70), whereas S/I liver is 2.25. Again there is a strongly significant difference between S/I liver and S/I of the two reference organs (small intestine and bone marrow). That part of S liver which is supposed to be due to syngenesio-transfused cells is estimated to be equal to S liver — S/I reference organs $\times$ I liver. That part of the relative activity in the spleen which is due to syngenesiotransfused cells is practically the same whether the calculations are based on S/H or S/I (21.80 and 19.69 respectively). (See Fig. 5.)

The question on page 40 can thus be answered affirmatively. It can be said that part of the activity of the syngenesiotransfused lymphocytes has been found in the spleen and liver after 24 hours, and part of the activity of the heterotransfused lymphocytes has been found in the liver.

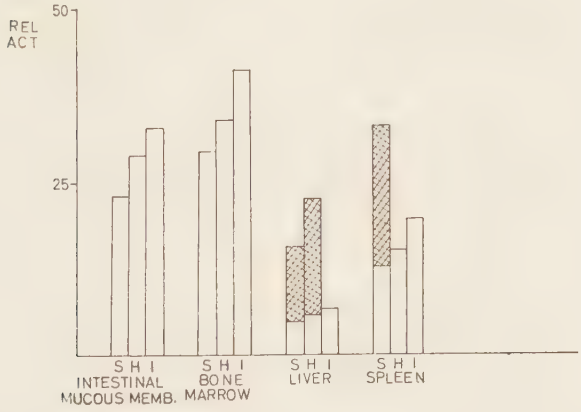

Fig. 5. Interpretation of the results of experiments 1 and 2. Those parts of the rel. act. which are supposed to be due to syngenesio- and heterotransfused lymphocytes are shown cross-hatched.

TRANSFUSION OF LABELLED LYMPHOCYTES IN RATS WITH PERITONITIS

Experiment 3.

Like the transfusion in normal rats, this experiment was based on the hypothesis that syngenesiotransfused cells do function in the receptor, but that heterotransfused do not. Heterotransfusions were again used as controls. The value of the transfusion experiment in normal animals was entirely dependent on whether the syngenesiotransfused lymphocytes did function in the receptor or not, and a test of function was sought for.

In acute inflammation the polymorphonuclear cells that leave the circulation form the chief cellular constituents of the early exudate. The mononuclear phagocytes or macrophages increase in number later. There are essentially two opinions on the genesis of the macrophages: one view is that the macrophages are derived from fibrocytes in situ, and some other workers assert that most of the macrophages are derived from circulating lymphocytes and monocytes. The reader is referred to the monographs by Kolouch (1939) and Menkin (1940). If the second view is correct it should be possible to test the function of the syngenesiotransfused cells by producing a mononuclear exudate in the receptor. Do the syngenesiotransfused cells take any part in the formation of such a mononuclear exudate? Or is there a larger relative content of P^{32} in the exudate cells after syngenesio- than after hetero-transfusion? There is much to cast suspicion on the view that the lymphocytes are transformed into macrophages (see page 11): an experiment to test the above question would be the more justified if it could also be used to confirm the role of the spleen in taking up the labelled lymphocytes, and to compare the distribution of $DNAP^{32}$ in the right and left sides of the liver.

Procedure. Peritonitis was produced in 3-month-old rats of about 145 g, by intraperitonal injection of 10 cc. liquid paraffin (Chase 1945). After 2 days syngenesio- and hetero-transfusions of labelled lymphocytes were performed as described in experiment 1, except that in this experiment the lymphocyte suspension was injected into the saphenous vein. 14 syngenesio- and 15 hetero-transfusions were carried out. After a further 24 hours the animals were killed, and

the DNAP^{32} from the right and left sides of the liver, and from the lung and spleen, was determined, and expressed in units of rel. act. By the rel. act. of a certain organ is here meant the number of impulses/minute/mg. DNAP from the organ in question % of the sum of the number of impulses/minute/mg. DNAP from right and left sides of the liver, lung, and spleen.

The exudate, containing about 70 % mononuclear cells, was collected with a spoon from the peritoneal cavity, which in addition was rinsed with normal saline. The diluted exudate was centrifuged (1000 r./min. for 10 min.), and the supernatant fluid poured off. 10 cc. of an ice-cold solution of 7 % tricloracetic acid was added to the remainder, and this preparation was stirred for 20 minutes. The exudate was washed once more in another 10 cc. of the trichloracetic acid solution. After pouring off the acid solution, 1.5 cc. of H_2SO_4 was added to the rinsed cells, and the content transferred to a Kjeldal flask and wet-ashed. The number of impulses/mg P of this mixture of organic P from the cells of the exudate was determined, and expressed in units of rel. act. By rel. act. in this case is meant the number of impulses/minute/mg organic P % of the same sum as above.

The results are described in Fig. 6 and Table 4 (Chapter X).

The quotient S/H is approximately the same in both sides of the liver and lung (0.39). S/H spleen, on the other hand, is 2.71, and there is a strongly significant difference between this quotient and each

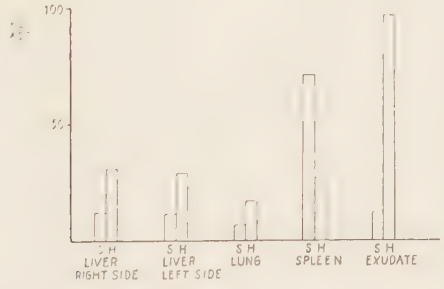

Fig. 6. Distribution of DNAP^{32} after syngenesiotransfusion (S) and heterotransfusion (H) of radioactively labelled lymphocytes in rats with peritonitis. The heights of the columns indicate the rel. act. of DNAP. (Right side of the liver + left side of the liver + lung + spleen = 100.) In the case of the exudate cells the columns indicate the rel. act. of org. P; see text.

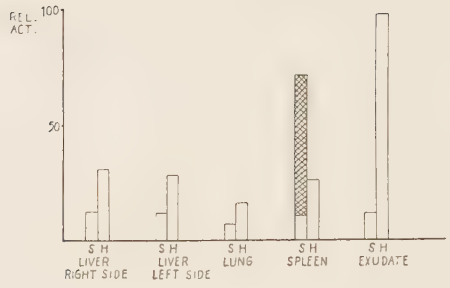

Fig. 7. Interpretation of experiment 3. That part of the rel. act. that is supposed to be due to syngenesiotransfused lymphocytes is shown cross-hatched.

quotient for the reference organs (viz. the two sides of the liver and the lung). Again, a certain part of S spleen is supposed to be due to syngenesiotransfused cells. (See Fig. 7.)

Unfortunately the mononuclear exudate was too scanty for accurate determination of $DNAP^{32}$. Instead, the calculations were based on the total non-acid soluble P. This implies that the S/H exudate cannot be compared with the remaining S/H. Nevertheless, the results suggest that it is very improbable that S-cells have been transformed into exudate cells to a larger extent than H-cells have, but whether or not any such transformation really takes place cannot be decided. There was no difference between the concentrations of $DNAP^{32}$ in the right and left sides of the liver, either after syngenesio- or hetero-transfusion.

A hasty glance at the results of experiments 1 and 3 shows that the distribution of $DNAP^{32}$ between the spleen and the reference organs has shifted to the advantage of the spleen in the case of syngenesiotransfusion in rats with peritonitis as compared with syngenesiotransfusion in normal rats. With regard to the suggested importance of the lymphatic system, including the spleen, in inflammations, this observation warrants further discussion. In both experiments the liver, lung, and spleen were examined for $DNAP^{32}$. In comparing the findings the calculations will be restricted to include only these organs. The rel. act. of an organ is defined as the number of impulses/minute/mg DNAP from the organ in question in % of the sum of the number of impulses/minute/mg DNAP from liver, lung, and spleen. In the rats with peritonitis the arithmetic mean of the values of the right and left sides of the liver is used.

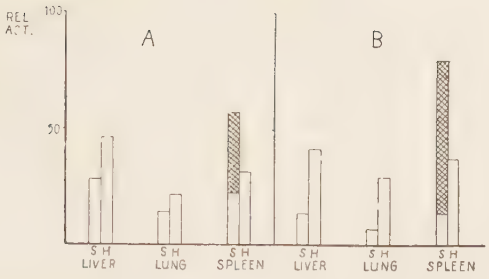

Fig. 8. The distribution of $DNAP^{32}$ after syngenesiotransfusion (S) and heterotransfusion (H). The heights of the columns indicate the rel. act. (liver + lung + spleen = 100). A. Transfusion to normal rats; B. to rats with peritonitis. The rel. act. supposed to be derived from syngenesiotransfused cells is shown cross-hatched.

The result of such a calculation is evident from Fig. 8 and Table 5 (Chapter X).

In the case of the liver the difference between S/H in healthy and diseased animals is statistically significant, and in the case of the lung the difference between these quotients is almost significant. To estimate the degree of significance shown by liver and lung together concerning the differences in S/H, a weighed critical ratio was calculated (viz. the sum of the critical ratios for liver and lung divided by $\sqrt{2}$). This showed that the difference between S/H reference organs in healthy and diseased animals was strongly significant. It has thus been shown that a larger portion of the syngenesiotransfused lymphocytes was found in the spleen in diseased than in healthy animals, heterotransfusions being used as controls.

INTERPRETATION AND DISCUSSION OF THE RESULTS

The suspension of radioactively labelled lymphocytes contains a relatively small quantity of P^{32}, combined in the cells as $DNAP^{32}$. The P^{32} of the suspension consists mainly of inorganic P^{32} and P^{32} associated with the cells in another, less stable form. To make the discussion clearer this larger fraction of P^{32} is called 'free P^{32}'. The radioactivity measured in the different samples is thus mainly $DNAP^{32}$ deriving from free P^{32}. The three possible fates of the lymphocytes, mentioned on page 35, will now be discussed with regard to the results obtained.

1. Dissolution of the lymphocytes cannot be excluded. The $DNAP^{32}$ then set free increases that part of the measured activity

which is due to free P^{32}. All the transfused cells are not dissolved, however: if this were the case distribution of $DNAP^{32}$ would be the same after syngenesiotransfusion, or heterotransfusion, or injection of inorganic P^{32}.

2. Can the results be explained as an effect of the reticulo-endothelial system? In experiment 1 S/H spleen differed from S/H reference organs. It is possible that both S-cells and H-cells were taken up by the RES, the H-cells rapidly being destroyed but the S-cells still detectable in the RES of the spleen 24 hours after injection. This explanation can be ruled out in two ways: i. The postulation that the H-cells were rapidly destroyed was shown to be wrong — in experiment 2 H-cell-activity was found in the liver; ii. it should also be possible to detect the S-cells by means of deviating quotients in other RES organs such as liver, bone marrow, lymph nodes, and lungs (Persson 1952, Gabrieli 1951). It may be argued that part of the rel. act. of the reference organs derives from cells taken up by the RES in both kinds of transfusion. Besides the fact that the RES of the spleen is not dominant to such a large extent among the organs of the RES, it is very improbable that the S/H quotients are so uniform for all organs of the RES but the spleen. Thus the result of experiment 1 cannot be explained as a pure effect of the RES. A similar argument can be applied to the result of experiment 2. H/I liver differs from H/I reference organs. This cannot be due to a pure effect of the RES. If it were, the H/I of the RES organs (bone marrow and spleen) would, like H/I liver, differ from H/I small intestine. Further, S/I liver and S/I spleen differ from S/I small intestine and S/I bone marrow. Again, if the result were due to the RES, the S/I of the three RES organs should differ from S/I small intestine (this, unfortunately, was the only organ examined which did not belong to the RES). What has been said about the results of experiment 1 applies to experiment 3.

Gabrieli (1951), who studied the function of the RES by means of radioactive chromium phosphate, found that when the injected particles were coarse the lungs absorbed the larger part of the colloid, whereas if the dispersion were finer the liver took up more. In Gabrieli's experiments the spleen absorbed equal amounts of the coarse and the fine dispersion. Even if it is supposed that the spleen has a very high attraction for still coarser dispersions my results cannot be explained in this way. Gabrieli further demonstrated a significant transportation of the chromium phosphate within the

RES after the administration of highly radioactive suspensions, but no transportation was demonstrable after the injection of less active suspensions. In my transfusion experiments the concentration of P^{32} in the RES was far less than in Gabrieli's experiments, and a disturbing effect of transportation within the RES can also be excluded from other sides.

3. The last of the three possible fates of the injected cells is that they behave like the lymphocytes (viz. the newly formed thymocytes) of the receptor. This hypothesis is supposed to be valid for both types of transfusions until the results can be reasonably explained in another way.

Björkman (1947), who investigated the sinus filter of the spleen in rabbits (Knisely 1936, Fåhraeus, Bergenhem 1936), injected intravenously a rice-starch suspension containing grains of various size, and observed the location and dimensions of these grains in different parts of the spleen. He found that the mean diameter of the grains in the sinuses was larger (3.51 ± 0.02 μ) than that of the grains in the pulpcords (2.70 ± 0.01 μ). The sinus wall evidently acts as a filter which retains larger particles from a suspension of the above kind, and allows smaller ones to pass. The results of my transfusion experiments cannot, however, be explained by the filtering of the cells in the spleen.

The experiments done to localize transfused lymphocytes are summarized in Table 1.

The results of Osogoe and Fichtelius are closely similar: the investigations can be said to complement each other. Weisberger's conflicting result has already been discussed (page 21). In Farr's experiment the transfused lymphocytes were found in the bone marrow and lymphoid tissue. It is possible that differences in the results are due to differences in the labelling. In my experiment the result depends on newly formed lymphocytes: but during staining it is not known which cells are selected so that other lymphocytes may influence the result.

The result of my transfusion experiments raises the question of whether there are any observations to condemn or support the hypothesis that the lymphocytes are removed from the blood in the spleen or the liver. In an investigation on the remote effects of removal of the normal spleen in man (Ask-Upmark 1935) it is asserted that the increased number of mononuclear cells in the dif-

Table I. Comparison of the experiments of Osogoe,

	Receptor animal	Type of transfusion	Origin of transfused lymphocytes	Label
Osogoe 1950	Rabbit, 600—2 400	Homoiotransfusion	Mesenteric lymph nodes: rabbit, 6 months old	No Label
Farr 1951	Rabbit, age?	Autotransfusion	Popliteal lymph nodes: rabbit, ? months old	Non-toxic vital stain *in vitro*
Weisberger *et al.* 1951	Rat, 150—200 g.	Homoiotransfusion	Intestinal lymphatics: rat, 200—300 g.	Large doses P^{32} *in vivo*
Fichtelius 1953	Rat, 3 months old	Syngenesio- and heterotransfusion	Thymus: rat, 3 months old	Large doses P^{32} *in vivo*

ferential count seems to be the most constant of the changes in blood morphology. The interpretation of this finding is very difficult: a lymphocyte-producing organ has been removed (Andreasen and Ottesen 1945. Snook 1946). hypertrophy of the remaining lymphoid tissue is induced (Warthin 1902). and the control of the level of blood lymphocytes may change. The white blood corpuscles are more numerous in the splenic vein than in the artery, and mononuclear leucocytes appear in great excess in the vein (Morris 1914). These are not the only lymphocytes to leave the spleen: there is a deep lymphatic plexus which pierces the white pulp, and opens into the lymphatic vessels of the hilus (Snook 1946). None of these observations are evidence against the hypothesis that the lymphocytes are removed from the blood in the spleen: it may be that some of the cells, already separated, return, and continue to the liver.

Preparation of lymphocyte suspension	Method of identifying the lymphocytes	Method of control	Interval after transfusion, and organs where lymphocytes were recovered
Cut into small pieces in 0.9% NaCl, gently stirred, filtered through gauze, washed once by centrifuging, filtered once more	Examination of histological sections	Injection of killed and injured cells	24 hours: liver 48 and 72 hours: liver, spleen
Cut into small pieces in Tyrode solution, filtered through gauze, labelled with stain in Tyrode for an unspecified length of time	Examination of histological sections	Heterotransfusion	2 hours: bone marrow, lymphoid tissue 12 hours: bone marrow (lymphocyte→myelocyte), lymphoid tissue
Cannulation of lymph vessels for 4 hours, centrifuged once, washed twice by centrifuging	Determination of radioactivity per g. tissue	Injection of disintegrated cells and of inorganic P^{32}	10 minutes: lung, spleen
Cut into about ten pieces, 0.9% NaCl forcibly injected into the pieces, squeezed with forceps, debris allowed to sediment for 1 minute	Determination of radioactivity per mg. desoxyribose nucleic acid phosphorus	Injection of inorganic P^{32}	24 hours: Syngenesiotransfusion: spleen, liver. Heterotransfusion: liver

If the lymphocytes are removed from the blood in the liver some histological evidence of this is to be expected. It is, indeed, common to find slight lymphocytic infiltration in the periportal spaces of the liver, especially round the interlobular branches of the bile duct (Osogoe 1950), and it is very interesting to note that the degree of this lymphocytic infiltration may be increased markedly in the absence of inflammatory processes in the liver, for example after X-ray irradiation (Horri 1949, quoted by Osogoe 1950).

The difference between the behaviour of S-cells and H-cells might be explained by the different immunological states of the donors. The S-cells derive from donors with an acquired immunity to the same infections as the receptor, whereas the donors in heterotransfusion have been exposed to other antigens.

There is much well-known work indicating antibody formation in the spleen — especially if antigen is injected intravenously. As early as 1898 the works of Pfeifer and Marx, and Wassermann indicated some sort of relationship between

antibody formation and the spleen as expressed by the presence of high titres in the organ after injecting various antigens. The cell changes in the spleen during antibody formation have been studied by Huebschmann (1913), Bjørneboe and Gormsen (1943), Fagreus (1948),and others, all of them drawing attention to the abundant plasma cell proliferation.

In the rats with peritonitis a slight non-specific secondary response can be expected (Freund 1953) with increased production of all antibodies normally produced by the receptor, and with proliferative changes in the spleen. The S-cells are found to a larger extent in the spleen in rats with peritonitis than in normal rats. These findings accord well with the above hypothesis: the divergence between the behaviour of the S-cells and the H-cells increases when the antibody formation is intensified. It is possible that some of the blood lymphocytes normally disappear in the spleen, and that their number is increased during acute splenic swelling, the lymphocytes being transformed into the acute splenic tumour cells of Rich, Lewis, and Wintrobe (1939), or into the transitional cells of Fagreus (1948), and developing at least into mature plasma cells. Kelsall (1949), who used hamsters, states that about 3% of the white blood corpuscles in the splenic and portal veins consist of plasma cells although none of these could be demonstrated in the jugular vein. Her findings suggest that some of the S-cells recovered in the spleen develop into plasma cells, and that some of them are transported to the liver (where they were also found in my transfusion experiment?). If it is assumed that some of the lymphocytes are transformed into plasma cells, and during this development release antibodies, surprisingly many of the apparently contradictory opinions about the site of antibody formation can be reconciled.

There are very few plasma cells in the thymus (Fagreus 1953), and no antibody formation can be shown (Harris, Rhoads, and Stokes 1948). In lymph nodes and spleen, on the other hand, there are numerous plasma cells, and the antibody formation has been clearly demonstrated (Fagreus 1948, Ehrich, Drabkin, and Forman 1949). Only slight evidence of antibody formation in the spleen appeared on subcutaneous injection of antigens, but intravenous injection was followed by high antibody titres in splenic extracts (Harris, Rhoads, and Stokes 1948). Similar results were obtained by Rowly (1950), who found, in splenectomized rats, little or no antibody formation when small amounts of antigen were injected into the blood-stream. In contrast, when antigen was injected intraperitoneally, intradermally, or intraportally, antibody-formation was quite as strong as in normal rats. The antibody formation was not influenced by thymectomy (Harris, Rhoads, and Stokes 1948) but unfortunately the antigen was administered subcutaneously in his experiment.

The above remarks and the result of the transfusion experiments suggest a relationship between thymus and spleen. Together they may constitute a large, central 'lymph node' — 'a haemolymph node', if you will. The thymus produces lymphocytes and the spleen forms the antibodies with the aid of them. Perhaps it is more than chance that both organs appear at the same time in phylogenesis (Bargmann 1943, Hartmann 1930). In the lymph nodes, on the other hand, the production of lymphocytes and antibodies is combined in one organ.

Some of the transfused cells have been found in the liver. Here the enzyme and the protein content of the lymphocytes might be of importance but our knowledge in this is still to inadequate to permit even of a venture into speculation.

CHAPTER VII

LABELLING AND TRACING OF LYMPHOCYTES WITHIN THE SAME ANIMAL

THE APPEARANCE OF LABELLED LEUCOCYTES IN THE BLOOD

Ottesen (1948), on the pattern of Shemin and Rittenberg (1946), tried to determine for how long the white blood cells of a hen remain in the circulating blood. He injected radioactive phosphorus at the beginning of the experiment. The phosphorus was then built into, for example, the DNA of the leucocytes, and remained there until the cells decayed (see page 24). He took blood samples from the animal at different times, and isolated the mixture of leucocytes by ordinary centrifuging. The specific radioactivity of the DNAP in the blood corpuscles, and of inorganic P in blood plasma, was then determined. Specific radioactivity is here defined as the number of impulses /mg DNAP, (or /mg inorganic P) in % of the injected dose. On the basis of these values Ottesen calculated the percentage of leucocytes formed during the first day of the experiment and present in the blood at the time of the taking of the sample. Similar experiments have been done in rabbits, but the lymphocytes were separated from the other white blood corpuscles (Fichtelius 1951). The separation of the lymphocytes, and the subsequent determination of DNAP, craved large amounts of blood, and thus the animals were bled only once at different times after the injection of P^{32}.

Some differences between my results and Ottesen's led to further investigations. The interpretation of these, however, demands still more experiments. They will be briefly mentioned, but not discussed, in this paper, and should be looked upon as preliminary experiments to the one described in page 58 onwards.

THE APPEARANCE OF LABELLED LEUCOCYTES IN THE BLOOD OF RABBITS AFTER REPEATED VENEPUNCTURE

Experiment 4.

In my rabbit experiments the labelled cells seem to appear biphasicially in the blood, whereas in Ottesen's hen experiments the

cells enter the blood following a one-top curve. How can this be explained?

In the rabbit the lymphocytes predominate numerically in the blood, constituting 75 % of the leucocytes (Sjövall 1936). The same two-top curve is found whether the calculations are based on isolated lymphocytes or on a mixture of leucocytes. In the hen there is a similar predominance of lymphocytes — 64 % according to Dukes (1943). Thus the experiments do not differ in this respect. Ottesen, however, used only one hen, and had to bleed it many times for the repeated determinations of $DNAP^{32}$ in the leucocyte mixture. In my experiment the rabbits received only the initial injection before being killed, and the actual samples taken. It is possible that the repeated bleeding influenced Ottesen's result. The biphasic appearance of the lymphocytes in the blood has also been observed in a sheep (Fichtelius 1951): it is possible that the repeated bleedings (80 cc. 6 times before the second top) did not markedly affect this bigger (63 kg) animal. The problem thus posed by this experiment is whether the biphasic appearance of lymphocytes vanishes when rabbits are repeatedly bled after the initial injection of P^{32}.

Procedure. The experiment was performed on 5 adult, male rabbits weighing between 3.2 and 4.2 kg. At the beginning of the experiment 100 μC per kg body-weight was injected intravenously. On the 3rd, 6th, 9th, 12th, and 15th days after this injection about 30 cc. of blood was collected from the auricular veins by means of a vacuum receiver devised by Sjövall (1936). A mixture of leucocytes was isolated from the heparinized blood by destroying the red blood corpuscles and thrombocytes, and subsequent centrifuging (see Scilard 1923). The $DNAP^{32}$ of the leucocytes was determined by the method described above, and the spec. act. calculated as the number of impulses/minute/mg DNAP, % of the injected dose (the dose being expressed as the number of impulses/minute/g body-weight).

The result is shown in fig. 9.

No increase in spec. act. was observed in 4 of the 5 animals. Thus the absence of a second top in Ottesen's experiment may very well be explained by the bleedings, either *per se* or by some factor connected with them.

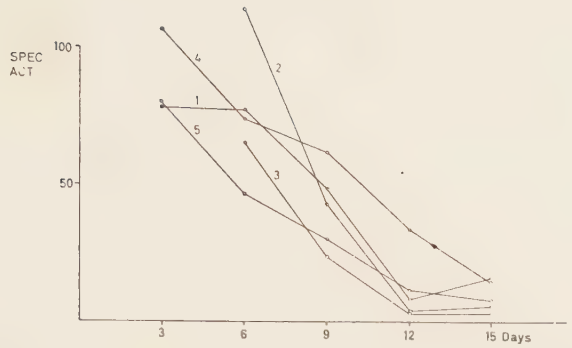

Fig. 9. Rabbits 1—5. Ordinate: spec. act. of DNAP from leucocytes. Abscissa: time in days.

BIPHASIC APPEARANCE OF LABELLED LEUCOCYTES IN THE BLOOD OF RABBITS BLED TWO OR THREE TIMES

Experiment 5.

Since the question put in experiment 4 has been answered affirmatively, the results previously obtained (Fichtelius 1951) become open to doubt. These results must be considered uncertain because of the great errors of estimation, and individual variations, combined with the small series. The great individual variations can be avoided by a compromise between the earlier investigation and the one just described. The problem now is, whether it is possible to obtain a two-top curve when the rabbits are bled only two or three times. The experiment was divided into three parts. Firstly (5 a): Is the spec. act. of leucocyte-DNAP in the same animal higher on the 3rd than on the 6th day after the initial injection, or is there a maximum of activity before the 6th day? The next question is common to experiments 5 b and 5 c: Is it possible to show an activity increase in the same animal after the 5th or the 7th day following the initial injection of P^{32}.

Procedure. Experiment 5 a. The experiment was carried out on 16 adult male rabbits of 2.5—4.4 kg. At the beginning of the experiment 100 μC P^{32} per kg body-weight was administered intravenously. On the 3rd and 6th days 30 cc. blood was let, and the spec. act. of DNAP from the leucocytes measured as in experiment 4. *Experiment 5 b and 5 c.* 11 male rabbits weighing 2.9—3.7 kg were used. The same dose of P^{32} as in experiment 4 and 5 a was administered in the

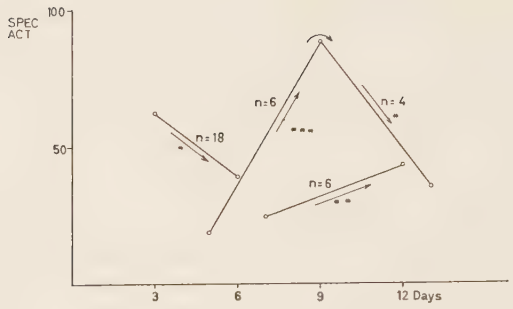

Fig. 10. Rabbits 6—33. Ordinate: spec. act. of DNAP from leucocytes. Abscissa: time in days.

same way. Blood was taken on the 5th, 9th, and 13th, and the 7th and 12th days, respectively, and the $DNAP^{32}$ determined as above.

The result of the experiment is indicated in Fig. 10 and Table 6 (Chapter X).

Experiment 5 a shows a significant decrease in the spec. act. from the 3rd to the 6th day (Student's test, one-tailed, applied). When logarithms are used in the calculation (to standardize the deviation of the observation) the decrease is almost significant (*).

Experiment 5 b. There is a strongly significant (***) increase in the activity from the 5th to the 9th day (Pitman's test, one-tailed, applied — Kendall 1948). Using the same method of calculation there is an almost significant decrease from the 9th to the 13th day.

Experiment 5 c. The application of Student's test, one-tailed, gives a significant (**) increase from the 7th to the 12th day, both with the standard test and with the use of logarithms.

These three experiments give cause for some reflection. Firstly, it is surprising to find only a nearly significant decrease in the first experiment in spite of using 18 animals. The value for the 6th day is also suspiciously high compared with the values for the 5th and 7th days. Secondly, it is remarkable that the second top has moved from the 15th to the 9th day (see Fichtelius 1951). Finally, the increase is less marked between the 7th and 12th days than between the 5th and 9th days. These facts may also be due to the bleeding.

Experiment 6.

Like experiment 5, this experiment was made in attitude of doubt as to the biphasic appearance of lymphocytes in the blood (Fichtelius 1951). Indeed, the result of the sheep experiment seemed to corroborate the results obtained in the rabbit experiment, but nevertheless only one sheep was examined. Is it possible to obtain a biphasic appearance of labelled leucocytes in other big animals not appreciably influenced by the magnitude of the repeated bleedings necessary?

Procedure. At the beginning of the experiment 50 μC of P^{32} per kg body-weight was injected subcutaneously in 3 goats weighing 45, 29, and 26 kg respectively. On the 3rd, 6th, 8th, 11th, and 15th days 40 cc. of blood was let. The leucocytes were separated from the heparinized blood with the aid of simple centrifuging. The buffy coat was gently lifted out and washed once in normal saline containing 0.02 % saponin. The $DNAP^{32}$ from the leucocytes was measured as above.

The result can be seen from Fig. 11.

In the largest animal (45 kg) (which was hardly influenced by the magnitude of the venepuncture) a marked minimum in the activity is obvious, and it is followed by an increase on the 15th day. In the smaller animals the second top leucocytes have reached the blood earlier, and the minimum of activity has disappeared. Only a slight

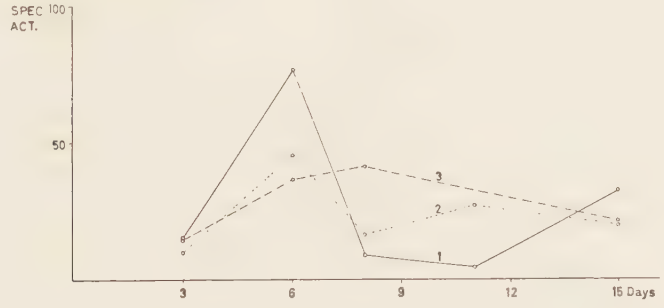

Fig. 11. Goats 1—3. Ordinate: spec. act. of DNAP from leucocytes. Abscissa: time in days.

increase can be seen on the 12th day in goat 2. These results give further support to the view that the bleeding influences the appearance of the labelled cells in the blood. It seems as if the results depend on the relative amount of blood let.

FURTHER NOTES ON THE APPEARANCE OF LABELLED LEUCOCYTES IN THE BLOOD OF TWICE-BLED RABBITS

Experiment 7.

The results of experiment 5 and 6 support the view that venepuncture produces the second top in the blood earlier than if the animals were not bled. It has been seen that this second top is more marked when 4 days are allowed to elapse between samplings than when the interval is 5 days. The following question now arises: Is an interval of 3 days between samplings enough to produce the second top of labelled cells before the time expected if the animals had not been bled?

Procedure. 100 μC per kg body-weight was injected intravenously in 19 male rabbits weighing 2.7—4.0 kg. Blood was collected on the 6th and 9th days. The procedure was otherwise as in experiments 4 and 5.

The results are evident from Fig. 12 and Table 6 (Chapter X).

No significant difference was found between the activities on the 6th and 9th days (the interval —1.8—+12.3 includes the true

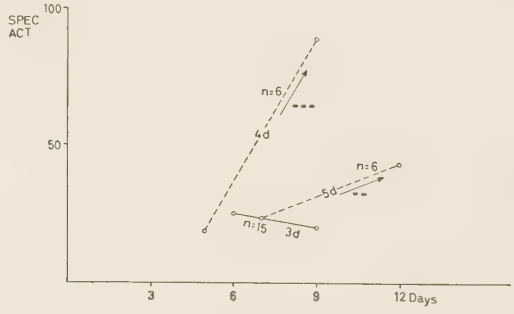

Fig. 12. Rabbits 34—53 (———). Ordinate: spec. act. of DNAP from leucocytes. Abscissa: time in days. The results of experiments 5 b and 5 c are indicated (— — —).

mean of the difference with a confidence of 95 %). It seems that an interval of 4 days between the samplings gives the largest increase, 5 days produced a lesser increase, and an interval of three days is too short to have any effect.

THE RESULTS OF EXPERIMENTS 4, 5, AND 7 COMPARED

It may be of interest to make the following calculations and comparisons from the material described in the previous sections. See Fig. 13 and Table 7 (Chapter X).

Blood was let on the 6th day both from rabbits which had not previously been bled, and from animals bled on the 3rd day. The spec. act. was higher in the latter animals, and the difference was significant (Behrens-Fisher's test, one-tailed, applied — Kendall 1948). This is well in keeping with the earlier statement that only near significance was obtained when the rabbits were bled on the 3rd and 6th days. It is worth noting that an interval of 3 days at the beginning of the experiment is enough to produce an activity increase, whereas later on the same interval is too short.

Blood was let on the 9th day from 3 groups of rabbits: 1. bled on the 6th day; 2. bled on the 5th day; and 3. bled on both 3rd and 6th days. The spec. act. was highest in the animals that were bled 4 days earlier, and higher in animals bled on the 3rd and 6th days than in those bled on the 6th day only. The significance of the differences was calculated by Pitman's test, one-tailed, applied, and is indicated in Fig. 13. The results illustrate well the fact that the inverval of 3 days was too short to have any effect.

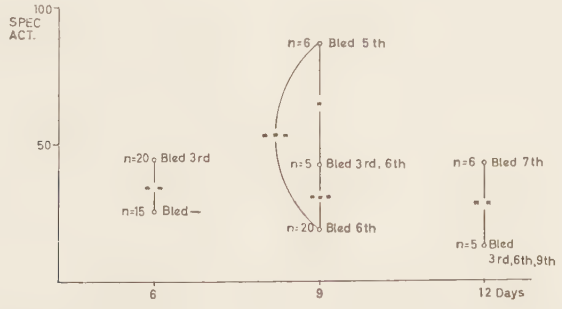

Fig. 13. Comparisons based on the results of experiments 4, 5, and 7. Ordinate: spec. act. of DNAP from leucocytes. Abscissa: time in days.

The rabbits from which blood was let on the 12th day can be divided into 2 groups: 1. those bled on the 7th day; and 2. those bled on the 3rd, 6th, and 9th days. The activity on the 12th day was lower in the second group than in the first, and the difference was significant (Pitman's test, one-tailed, applied). A reasonable explanation is that when the rabbits have been bled so many times the second-top leucocytes have already left the blood on the 12th day. In addition, many of the labelled cells have been removed by blood-letting.

TRACING OF SECOND-TOP LEUCOCYTES

Experiment 8.

The biphasic appearance of the labelled leucocytes and the influence of bleeding offer much ground for speculation. However this may be, it is a fact that labelled leucocytes can be induced in the blood 4 days after a venepuncture, a time when they otherwise do not appear in the blood: and it is probable that this accumulation of labelled cells consists to a large extent of lymphocytes. This assumption is based on the similarity of the 'appearance curves' of isolated lymphocytes and of the leucocyte mixture (Fichtelius 1951). It should thus be possible to label and trace the lymphocytes within the same animal. We know that the blood span of the lymphocytes is very short. If, then, radioactively labelled lymphocytes accumulate in the blood to a maximum extent at a particular time, it follows that a maximum number must also leave the blood simultaneously: the possibility of demonstrating them in the organ through which they leave the blood is then maximal.

In the following experiment the second-top leucocytes were induced by a venepuncture. When the second-top was estimated to be maximal the animals were killed and examined for radioactivity in lymph nodes, spleen, and liver. They were then compared with controls which had not been bled. More or less conjecturally the following apply to the organs examined: 1. Some of the lymphocytes are formed in the lymph nodes. The transfusion experiments did not suggest that any lymphocytes collect in the lymph nodes; these were thus considered as purely lymphocyte-producing organs in this experiment. If the lymphocytes constituting the second top were derived from lymph nodes the activity of these organs would

be lower in bled animals than in animals not bled. 2. No lymphocytes are formed in the liver, but the transfusion experiment showed this to be a possible receptor organ. The liver is here taken to represent a pure lymphocyte receptor. Some of the second-top lymphocytes may reach the liver when the animals are killed, in which case the activity of that organ would be higher in animals bled than in the controls. 3. Some of the lymphocytes are formed in the spleen, and some of the syngenesiotransfused cells were shown to be taken up there. In this experiment the spleen represents an organ which is both a producer and receptor of lymphocytes. Conditions in the spleen are thus more complex, and the splenic activity can be expected to be about the same in both the experimental animals and the controls. The question posed in this chapter is, 'What is the distribution of $DNAP^{32}$ in these "lymphocyte-producing" and "lymphocyte-receiving" organs in animals in which second-top leucocytes are induced in the blood, compared with the distribution of $DNAP^{32}$ in control animals?'

Procedure. The experiment was performed on 24 male rabbits weighing between 2.3 and 3.6 kg. At the beginning of the experiment 100 μC P^{32} per kg body-weight was injected intravenously. On the 4th day of the experiment 30 cc. of blood was let from 14 of the animals, the remaining 10 serving as controls. On the 8th day the animals were killed, and the number of impulses/minute/mg DNAP isolated from lymph nodes, spleen, and liver, was measured. The rel. act. of an organ was calculated as the number of impulses/minute/mg DNAP from the organ in question in % of the sum of the number of impulses/minute/mg DNAP from lymph nodes, spleen, and liver.

Results: The distribution of $DNAP^{32}$ among the organs examined was very similar in both series. No statistically significant difference could be shown. See Fig. 14 and Table 8 (Chapter X).

Our knowledge of the second-top leucocytes is inadequate. Even though most of them are lymphocytes it is possible that they behave in quite another way than the newly-formed thymocytes used in the transfusion experiments. It is not known, for instance, whether they are present in the blood for the first, second, or third time. The assumptions on which experiment 8 was based are too vague: a similar experiment might increase our knowledge if more organs were examined.

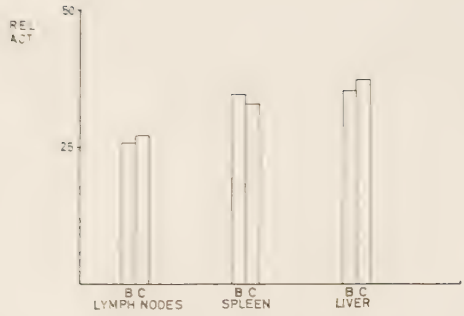

Fig. 14. Distribution of DNAP^{32} 8 days after the injection of inorganic P^{32}. The heights of the columns indicate the rel. act. (lymph nodes + spleen + liver = 100). B. Rabbits bled on the 4th day after injection. C. Controls not bled.

The results of the preliminary experiments (4—7) are more interesting than the negative result of the experiment to which they lead. I wish only to mention the demonstration of the second-top of leucocytes and the very strong influence of the venepunctures on it. It might be possible to elucidate the mechanism of the post-haemorrhagic leucocytosis by means of the method devised.

CHAPTER VIII

SUMMARY

CHAPTER I. Abbreviations and definitions.

CHAPTER II. This work deals mainly with the problem of the disappearance of the lymphocytes from the blood.

CHAPTER III. Before *the literature on the fate of the lymphocyte* is summarized, some fundamental questions are discussed — the size; the developmental potency; the probability that a lymphocyte-circulation exists; and, finally, the identity of the lymphocytes from the thymus and the lymphatic organs.

Histological studies: The lymphocytes probably leave the blood stream (page 15). It has not been indisputably shown that lymphocytes leave the blood via the epithelium of the digestive or respiratory tract (page 15), or via the epidermis (page 16). The presence of lymphocytes in the bone marrow is discussed, and an objective method of identifying the lymphocyte is appealed for (page 17).

Histopathological studies: The methods used in studying the lymphocytes in inflammation are criticized (page 11). The danger of drawing analogies between bone marrow under conditions of maturation arrest and under normal conditions is emphasized (page 18).

Tissue culture: It is possible that the conclusions based on tissue culture studies apply to a small number of lymphocytes, but it seems improbable that they are pertinent to the majority of lymphocytes (page 19).

Transfusion of lymphocytes: In the last years transfusion experiments with labelled and unlabelled lymphocytes have been performed.

CHAPTER IV. Some data on *radioactive phosphorus*, P^{32}, are given (page 22). There is a very intense turnover of desoxyribose nucleic acid, DNA, in lymphocyte-producing organs. The P^{32} incorporated into DNA molecules during the formation of lymphocytes remains in the cells throughout their lifetime (page 24). P^{32} was thus chosen for labelling the lymphocytes. The radiosensitivity of the lymphocytes is discussed, and it is conceded that labelling of lymphocytes with radioactive isotopes is fraught with chance.

CHAPTER V. *The methods* of isolation and wet ashing of DNA and of determination of phosphorus and radioactivity were described earlier: they are here used with slight modifications. The standard deviation of the single determination of radioactivity per mg. DNAP is usually less than 9.6% of the observation.

CHAPTER VI. *Radioactively labelled lymphocytes were transfused:* 1. from rabbit to rat — *heterotransfusion*; and 2. from rat to rat within the same litter — *syngenesiotransfusion*. The theory of acquired immunity as the cause of biological incompatibility has been accepted in this work (page 33). In these transfusion experiments the receptors were examined after 24 hours, and there would appear to be three possible fates for the transfused lymphocytes (page 35): 1. They may be dissolved, and their $DNAP^{32}$ set free and distributed throughout the body; 2. They may be taken up as antigens by the RES; 3. They may behave like the lymphocytes of the receptor, either after both transfusions or after syngenesiotransfusion alone.

P^{32} was administered to rabbits and rats respectively, and the DNA of the new lymphocytes was thus labelled. A suspension of lymphocytes was obtained from the thymus of the donors (page 35). The interval between the injection of P^{32} and the killing of the donor was determined, and the largest dose of P^{32} which could be injected bearing in mind the disturbing effect of radiation estimated (page 36). The labelled cells seemed to be destroyed *in vitro* irrespectively of their content of P^{32} (page 37).

Experiment 1. Is there any difference in the distribution of $DNAP^{32}$ among certain organs after syngenesio- and hetero-transfusion of lymphocytes labelled with P^{32}? The animals were killed 24 hours

after the transfusion, and the $DNAP^{32}$ from thymus, liver, mucous membrane of the small intestine, lymph nodes, bone barrow, lungs, and spleen was determined, and expressed in units of relative activity (page 38). *A certain part of the relative activity in the spleen after syngenesio-transfusion was found to be due to the transfused lymphocytes* (page 39), *and it can be said that syngenesio- and hetero-transfused cells are not taken up in the same way.*

Experiment 2. Is there any difference in the distribution of $DNAP^{32}$ among certain organs after transfusion of labelled lymphocytes, and after injection of inorganic P^{32}? After 24 hours the animals were killed, and the relative activity of DNAP from the mucous membrane of the small intestine, bone marrow, liver, and spleen, was calculated as in experiment 1. In both transfusions a certain fraction of the relative activity in the liver was found to be due to transfused cells (page 41). That fraction in the spleen due to syngenesiotransfused cells was practically the same whether the calculations were based on comparison with heterotransfusion or with injection of inorganic P^{32}. It can thus be said that *some of the activity of the syngenesio-transfused lymphocytes has been found in the spleen and liver after 24 hours, and some of the activity of the heterotransfused lymphocytes in the liver.*

Experiment 3. Transfusion experiments were performed on rats with aseptic peritonitis induced by liquid paraffin. Again, some of the relative activity in the spleen after syngenesiotransfusion was found to be due to transfused cells (page 44). It was surprising to find that *a larger number of the syngenesiotransfused cells was found in the spleen in diseased than in healthy animals, heterotransfusions being used as controls.*

The fate of the transfused lymphocytes is discussed. Dissolution of the cells cannot be excluded, but all of them are not destroyed (page 46): nor can the results be explained as an effect of the RES (page 46), or as a filtering out of the cells in the spleen (page 47). *Syngenesio- and hetero-transfused lymphocytes are supposed to behave like the lymphocytes of the receptor until the results can be reasonably explained in another way.* No definite evidence to condemn the hypothesis that the lymphocytes are removed from the blood in the spleen and in the liver has been found in the literature (page 47).

CHAPTER VII. *Labelled leucocytes can be induced in the blood 4 days after a venepuncture at a time when they otherwise do not appear in the blood* (*experiments 5—7*). It is probable that this accumulation of labelled cells consists to a large extent of lymphocytes. The blood span of the lymphocyte is very short. If, then, radioactively labelled lymphocytes accumulate in the blood to a maximum extent at a particular time, it follows that a maximum number must also leave the blood simultaneously: the possibility of demonstrating them in the organ through which they leave the blood is then maximal.

The question posed in *experiment 8* is, 'what is the distribution of $DNAP^{32}$ in "lymphocyte-producing" and "lymphocyte-receiving" organs in animals in which a top of labelled leucocytes is induced in the blood by venepuncture, compared with the distribution of $DNAP^{32}$ in control animals?' Lymph nodes, spleen, and liver were examined but the result was negative: no difference could be shown between the rabbits bled and the controls. The demonstration of the strong influence of blood-letting on the appearance of labelled leucocytes in the blood is more interesting than the negative result of the experiment to which it leads.

CHAPTER IX

ACKNOWLEDGEMENTS

The investigations were carried out at the Histological Department of the University of Upsala.

I am much indebted to my chief, Professor Martin Wrete, Head of the Department, who generously placed the resources of the Department at my disposal, and who followed the investigations with keen interest. For his stimulating encouragement, kindness, and friendship, I wish to convey to Professor Wrete my most cordial thanks. During the course of this work I have had the privilege of discussing many problems with Dr. Sven Brolin, of the same institute. His erudite criticism has been of great importance to me.

For technical assistance in the experiments I wish to express my appreciation and gratitude to Mrs. Eva Kikas: I am also grateful to Dr. Marcia Skogh, who has helped me with the correction of the manuscript, and to Dr. Gunnar Eklund, who has helped me with the statistical analyses. Finally, I wish to thank all members of the staff of the Histological Department.

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CHAPTER X

TABLES

TABLE 2. Distribution of $DNAP^{32}$ after syngenesiotransfusion and heterotransfusion of labelled lymphocytes. The numbers indicate the relative activity of DNAP.

Thymus	Liver	Small intestine	Lymph nodes	Bone marrow	Lung	Spleen
			Syngenesiotransfusion:			
10.1	6.4	11.8	20.8	30.2	13.2	51.6
25.0	15.9	25.6	8.1	35.9	14.3	22.6
23.4	10.7	30.2	15.9	38.7	8.8	20.4
32.9	18.8	18.9	9.1	46.1	5.4	16.2
43.0	16.4	29.5	12.9	28.5	6.8	25.5
23.6	12.8	12.0	6.4	16.3	—	58.9
12.9	16.6	22.7	12.3	17.3	2.3	43.3
14.1	8.7	17.0	8.4	21.5	4.9	52.8
74.2	13.7	30.2	5.8	23.2	6.9	32.9
21.9	20.2	18.9	11.1	21.3	6.3	39.6
29.1	7.0	42.5	14.5	36.8	9.6	13.7
18.1	8.9	28.5	9.1	32.0	4.1	30.6
25.1	16.5	21.8	11.0	43.7	8.8	18.0
16.6	17.7	19.8	5.5	28.4	5.2	34.0
10.4	14.0	14.0	8.7	39.3	—	32.7
10.7	8.5	32.0	5.5	13.6	2.7	45.9
23.5	41.0	15.7	5.5	21.2	8.4	22.1
414.6	253.8	391.1	170.6	494.0	107.7	560.8
$\bar{x} = 24.39$	$\bar{x} = 14.93$	$\bar{x} = 23.00$	$\bar{x} = 10.03$	$\bar{x} = 29.06$	$\bar{x} = 7.18$	$\bar{x} = 32.99$
$\varepsilon = 3.77$	$\varepsilon = 1.93$	$\varepsilon = 2.02$	$\varepsilon = 1.03$	$\varepsilon = 2.41$	$\varepsilon = 0.89$	$\varepsilon = 3.35$

Table 2 (cont.).

Thymus	Liver	Small intestine	Lymph nodes	Bone marrow	Lung	Spleen
			Heterotransfusion:			
37.0	14.4	37.6	9.6	32.3	—	15.7
32.0	11.8	34.9	9.6	37.5	10.4	15.8
28.4	15.0	29.7	33.6	47.8	20.0	7.5
33.1	16.2	30.0	17.8	36.3	8.1	17.6
42.0	11.3	33.7	11.4	39.3	7.3	15.7
43.9	16.8	41.3	12.6	32.2	8.8	9.8
30.6	12.6	36.3	10.1	42.1	8.8	9.1
21.9[1]	38.7[1]	19.1[1]	9.8[1]	34.0[1]	12.7[1]	8.1[1]
34.2	13.4	20.0	17.3	44.0	11.5	22.6
30.4	11.3	35.2	—	36.7	12.0	16.7
29.4	32.8	23.9	7.5	34.0	11.3	9.4
9.8[1]	28.5[1]	16.5[1]	30.4[1]	23.3[1]	7.9[1]	31.7[1]
61.1	16.0	34.9	14.3	30.9	6.4	18.1
37.2	23.0	33.5	7.6	32.6	8.0	10.9
22.9	47.1	15.9	12.3	24.0	12.9	13.3
26.4	22.8	32.8	6.2	34.9	5.7	9.5
37.7	26.0	22.6	7.7	35.9	9.8	15.5
30.1	35.0	28.4	8.4	25.2	9.1	11.3
22.2[1]	37.0[1]	15.5[1]	6.3[1]	30.3[1]	13.5[1]	17.2[1]
33.5	17.8	31.8	7.5	31.5	6.8	18.8
643.8	447.5	573.3	240.0	684.8	191.0	294.3
$\bar{x} = 32.19$	$\bar{x} = 22.38$	$\bar{x} = 28.67$	$\bar{x} = 12.63$	$\bar{x} = 34.24$	$\bar{x} = 10.05$	$\bar{x} = 14.72$
$\varepsilon = 2.30$	$\varepsilon = 2.40$	$\varepsilon = 1.79$	$\varepsilon = 1.70$	$\varepsilon = 1.40$	$\varepsilon = 0.77$	$\varepsilon = 1.72$
$\frac{S}{H} \pm \varepsilon_{\frac{S}{H}}$						
0.76 ± 0.13	0.67 ± 0.11	0.80 ± 0.09	0.79 ± 0.12	0.85 ± 0.08	0.71 ± 0.10	2.24 ± 0.31

[1] Haematoma at the site of injection.

Table 3. Distribution of $DNAP^{32}$ after injection of inorganic P^{32}. The numbers indicate the relative activity of DNAP.

Small intestine	Bone marrow	Liver	Spleen
32.3	36.1	8.6	22.9
34.7	39.8	7.3	18.2
26.5	43.8	5.2	24.5
30.4	37.0	7.6	25.1
30.1 [1]	42.8 [1]	5.6 [1]	21.5 [1]
30.2	44.5	5.6	19.8
35.6	37.3	4.7	22.4
36.5	45.0	8.0	10.5
36.5	37.3	5.2	21.0
44.8	35.3	6.6	13.3
35.7	30.1	9.5	24.6
31.2	47.5	7.5	13.8
31.4	47.1	4.9	16.6
24.0	50.8	8.0	17.2
35.1	45.5	5.5	13.8
495.0	619.9	99.8	285.2
$\bar{x} = 33.00$ $\varepsilon = 1.27$	$\bar{x} = 41.33$ $\varepsilon = 1.47$	$\bar{x} = 6.65$ $\delta = 0.39$	$\bar{x} = 19.01$ $\varepsilon = 2.01$
$\frac{H}{I} \pm \varepsilon_{\frac{H}{I}}$			
0.87 ± 0.06	0.83 ± 0.08	3.37 ± 0.41	0.77 ± 0.10
$\frac{S}{I} \pm \varepsilon_{\frac{S}{I}}$			
0.70 ± 0.07	0.70 ± 0.06	2.25 ± 0.32	0.59 ± 0.18

[1] Peritonitis.

TABLE 4. Distribution of $DNAP^{32}$ after syngenesiotransfusion and heterotransfusion of labelled lymphocytes in rats with peritonitis. The numbers indicate the relative activity of DNAP.

Liver, right side	Liver, left side	Lung	Spleen	Exudate
		Syngenesiotransfusion:		
8.8	6.3	7.1	77.8	18.6
10.3	11.9	5.7	72.1	11.7
12.2	12.3	5.6	69.9	15.2
14.0	12.2	4.6	69.2	7.9
8.3	7.2	7.3	77.2	1.7
5.4	5.6	1.2	87.8	0.2
11.7	9.9	6.5	71.9	9.9
10.8	11.9	2.9	74.5	6.0
11.3	11.1	3.6	73.9	8.3
12.4	12.1	8.0	67.5	10.5
13.9	14.7	3.7	67.8	4.3
21.3	20.7	9.9	48.0	37.6
140.4	135.9	66.1	857.6	131.9
$\bar{x} = 11.70$	$\bar{x} = 11.33$	$\bar{x} = 5.51$	$\bar{x} = 71.47$	$\bar{x} = 10.99$
$\varepsilon = 1.12$	$\varepsilon = 2.19$	$\varepsilon = 0.70$	$\varepsilon = 2.67$	$\varepsilon = 2.85$
		Heterotransfusion:		
23.7	26.7	17.7	31.9	58.7
24.0	21.6	17.7	36.7	42.6
27.2	25.6	21.6	25.6	73.0
24.6	24.8	18.2	32.4	134.7
43.5	28.2	13.4	14.9	290.9
28.7	18.2	14.2	38.8	36.0
30.2	22.3	20.8	26.7	66.2
31.3	36.7	11.0	20.9	16.6
29.0	30.1	12.0	28.8	17.7
34.6	32.8	12.0	20.7	368.7
33.2	15.7	23.8	27.2	29.4
28.2	36.1	8.6	27.1	19.2
36.9	35.4	6.7	21.0	158.2
32.6	34.3	16.2	16.9	51.1
427.7	388.5	213.9	369.6	1363.0
$\bar{x} = 30.55$	$\bar{x} = 27.75$	$\bar{x} = 15.28$	$\bar{x} = 26.40$	$\bar{x} = 97.36$
$\varepsilon = 1.45$	$\varepsilon = 1.82$	$\varepsilon = 1.34$	$\varepsilon = 1.89$	$\varepsilon = 28.87$
$\frac{S}{H} \pm \varepsilon_{\frac{S}{H}}$				
0.38 ± 0.04	0.41 ± 0.07	0.36 ± 0.06	2.71 ± 0.22	0.11 ± 0.04

TABLE. 5. The distribution of DNAP^{32} after syngenesiotransfusion and heterotransfusion in healthy rats and in rats with peritonitis. The numbers indicate the relative activity of DNAP.

Liver	Lung	Spleen	Liver	Lung	Spleen
Healthy rats					
Syngenesiotransfusion			Heterotransfusion		
57.3	11.8	30.9	41.0	15.7	43.3
14.9	4.7	80.4	54.7[1]	19.9[1]	25.4[1]
31.1	9.1	59.8	63.1	16.5	20.4
38.0	20.3	41.6	50.7	19.1	30.1
20.4	9.4	70.2	60.1	14.9	25.1
23.0	31.6	45.4	64.3	17.6	18.1
30.5	9.5	60.0	55.0	19.1	25.9
25.5	12.9	61.5	39.5	15.8	44.7
13.1	7.3	79.6	41.8[1]	11.7[1]	46.5[1]
26.7	3.6	69.7	61.4	21.1	17.5
33.7	14.0	52.3	28.3	29.9	41.8
46.6	13.3	40.1	28.2	24.1	47.7
26.9	22.0	51.0	65.1[1]	21.3[1]	13.6[1]
30.1	27.1	42.8	41.3	28.9	29.8
9.0	18.6	72.4	47.3	25.0	27.7
			32.8	21.4	45.8
			38.6	19.4	42.0
			35.2	47.1	17.7
			31.1	27.3	41.6
426.8	215.2	857.7	879.5	415.8	604.7
$\bar{x} = 28.45$	$\bar{x} = 14.35$	$\bar{x} = 57.18$	$\bar{x} = 46.29$	$\bar{x} = 21.88$	$\bar{x} = 31.83$
$\varepsilon = 3.24$	$\varepsilon = 2.08$	$\varepsilon = 3.94$	$\varepsilon = 2.92$	$\varepsilon = 1.79$	$\varepsilon = 2.68$

[1] Haematoma at the site of injection.

Table 5 (cont.).

Liver	Lung	Spleen	Liver	Lung	Spleen
Rats with peritonitis					
Syngenesiotransfusion			Heterotransfusion		
8.2	7.7	84.1	50.3	24.4	25.3
12.5	6.4	81.1	56.6	10.5	32.9
14.0	6.4	79.6	47.4	12.7	39.9
15.1	5.3	79.6	32.4	31.5	36.1
8.4	8.0	83.7	50.7	18.0	31.2
5.9	1.2	92.9	42.0	17.1	40.9
12.1	7.3	80.6	39.9	15.3	44.8
12.8	3.2	84.0	51.6	16.7	31.7
12.6	4.1	83.2	35.6	28.2	36.2
14.0	9.1	76.9	30.5	18.6	50.8
16.7	4.3	79.1	55.8	21.0	23.2
26.6	12.5	60.8	32.8	24.2	43.0
158.9	75.5	965.6	35.9	29.3	34.8
			29.6	22.9	47.5
$\bar{x} = 13.24$	$\bar{x} = 6.29$	$\bar{x} = 80.47$	33.7	23.7	42.6
$\varepsilon = 1.51$	$\varepsilon = 0.86$	$\varepsilon = 2.13$	624.8	314.1	560.9
			$\bar{x} = 41.65$	$\bar{x} = 20.94$	$\bar{x} = 37.39$
			$\varepsilon = 2.47$	$\varepsilon = 1.58$	$\varepsilon = 2.03$
$\frac{S}{H} \pm \varepsilon_{\frac{S}{H}}$	Healthy rats		$\frac{S}{H} \pm \varepsilon_{\frac{S}{H}}$	Rats with peritonitis:	
0.61 ± 0.08	0.65 ± 0.10	1.80 ± 0.20	0.32 ± 0.04	0.38 ± 0.05	2.15 ± 0.13

TABLE 6. The appearance of labelled leucocytes in the blood of rabbits. The numbers indicate the specific activity of DNAP isolated from leucocytes at different times after the injection of P^{32}.

3rd day	6th day	5th day	9th day	13th day	6th day	9th day
78.0	78.0	18.8	39.0	36.1	20.4	21.3
107.1	74.5	20.0[3]	174.3[3]	47.8	18.6	16.7
80.1	46.7	24.1	134.0	36.8	14.7	17.1
75.9[1]	29.8[1]	21.2	75.6	20.0	17.8	14.9
107.1	38.2	15.3	67.0	—	65.7	27.8
71.8	34.2	13.9	41.9	—	26.6	41.5
113.5	37.2	113.3	531.8	140.7	37.1	18.4
41.0	33.3				35.9	17.5
38.8[2]	42.9[2]	$\bar{x} = 18.9$	$\bar{x} = 88.6$	$\bar{x} = 35.2$	14.0	19.6
31.8	33.4				29.6	19.4
32.6	80.8	7th day	12th day		28.9	22.3
89.3	8.1				16.3	16.7
21.0	48.5	37.4	78.5		16.0	16.9
40.5	31.5	30.3	38.7		12.3	15.7
25.7	16.6	23.1	38.2		31.7	20.7
28.9	28.7	15.6	32.3		385.6	306.5
48.3	17.0	16.1	40.2		$\bar{x} = 25.7$	$\bar{x} = 20.4$
91.3	22.2	23.1	31.0			
1122.7	701.6	145.6	258.9			
$\bar{x} = 62.4$	$\bar{x} = 39.0$	$\bar{x} = 24.3$	$\bar{x} = 43.2$			

[1] Lesion of the spinal cord with acute retention of urine.

[2] Pregnancy.

[3] Chronic abscess abdominal wall .

TABLE 7. The appearance of labelled leucocytes in the blood of rabbits with special regard to the influence of venepuncture. The numbers indicate the specific activity of DNAP isolated from leucocytes at different times after the injection of P^{32}.

6th day		9th day		12th day
not bled	bled 3rd day	bled 5th day	bled 6th day	bled 3rd, 6th, 9th day
20.4	78.0	39.0	14.5[3]	8.5
18.6	114.3	174.3	11.1	4.1
14.7	65.3	134.0	21.3	3.3
17.8	74.5	75.6	16.7	34.0
65.7	46.7	67.0	17.1	11.9
26.6	29.8[1]	41.9	14.9	61.8
37.1	38.2	531.8	27.8	$\bar{x} = 12.4$
35.9	34.2	$\bar{x} = 88.6$	41.5	
14.0	37.2		18.4	
29.6	33.3		17.5	
28.9	42.9[2]		19.6	
16.3	33.4		19.4	
16.0	80.8	bled 3rd, 6th days	22.3	bled 7th day
12.3	8.1		16.8	
31.7	48.5		19.6	78.5
385.6	31.5	49.2	16.7	38.7
$\bar{x} = 25.7$	16.6	42.7	16.9	38.2
	28.7	24.3	9.2	32.3
	17.0	63.3	15.7	40.2
	22.2	30.6	20.7	31.0
	881.2	210.1	377.7	258.9
	$\bar{x} = 44.1$	$\bar{x} = 42.0$	$\bar{x} = 18.9$	$\bar{x} = 43.2$

[1] Lesion of the spinal cord with acute retention of urine.

[2] Pregnancy.

[3] Chronic abscess /abdominal wall/.

TABLE 8. Distribution of $DNAP^{32}$ 8 days after the injection of P^{32} in rabbits bled on the 4th day and in rabbits not bled. The numbers indicate the relative activity DNAP.

Lymph nodes	Spleen	Liver
Rabbits bled on the 4th day.		
36.4	26.3	37.2
36.7	33.7	29.6
25.8	27.8	46.5
27.4	28.5	44.1
35.0	31.9	33.1
19.5	32.8	47.7
33.3	39.5	27.2
27.8	33.1	39.1
32.3	35.9	31.8
29.6	38.3	32.1
27.3	38.3	34.4
26.0	37.1	36.9
31.4	46.5	22.1
28.5[1]	36.5[1]	35.0[1]
417.0	486.2	496.8
$\bar{x} = 29.79$	$\bar{x} = 34.73$	$\bar{x} = 35.49$
$\varepsilon = 1.27$	$\varepsilon = 1.43$	$\varepsilon = 1.93$
Rabbits not bled		
34.2	35.8	30.1
25.0	34.0	41.0
31.4	36.7	31.9
32.3	20.6	47.1
33.0	31.9	35.1
27.0[2]	39.6[2]	33.4[3]
27.7	29.5	42.9
28.1	41.5	30.4
33.1	30.8	36.1
29.9	27.5	42.6
301.7	327.9	370.6
$\bar{x} = 30.17$	$\bar{x} = 32.79$	$\bar{x} = 37.06$
$\varepsilon = 0.98$	$\varepsilon = 1.94$	$\varepsilon = 1.88$

[1] Dermatitis.

[2] Lymphadenitis.

CHAPTER XI

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